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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

#18
A.G.J.
3/9/98

In re Application of:
Dennis E. HALLAHAN *et al.*

Serial No.: 08/540,343

Filed: October 6, 1995

For: METHODS AND COMPOSITIONS FOR VIRAL ENHANCEMENT OF CELL KILLING

Examiner: S.D. Priebe

Group Art Unit: 1804

Atty. Dkt: ARCD:194/HYL

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BRIEF ON APPEAL

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BRIEF ON APPEAL

BOX AF
Hon. Asst. Commissioner for Patents
Washington, D.C. 20231

Sir:

This is in response to the final Office Action mailed on July 8, 1997, the Advisory Action mailed on October 22, 1997, and the Notice of Non-Compliance mailed February 2, 1998 regarding the above-captioned application. This replacement brief is due on March 2, 1998. No fees are believed to be due in connection with the filing of this replacement Appeal Brief; however, should any fees under 37 C.F.R §§ 1.16 to 1.21 be deemed necessary for any reason relating to the enclosed materials, the Commissioner is hereby authorized to deduct said fees from Arnold, White & Durkee Deposit Account No. 01-2508/ARCD:194/HYL.

I. REAL PARTY IN INTEREST

This application has been assigned to ARCH Development Corporation.

II. RELATED APPEALS AND INTERFERENCES

There are no pending appeals or interferences for related cases.

III. STATUS OF THE CLAIMS

Claims 1-7, 9, 12, 14, 16, 17 and 28-34 have been canceled, while claims 8, 10, 11, 13, 15, 18-27 and 35-55 remain pending in the application and stand appealed. Appendix I contains a summary of the pending claims.

IV. STATUS OF THE AMENDMENTS

The amendments offered in the response to the Final Office Action are entered by virtue of the filing of appellants' brief, as indicated in the Advisory Action of October 22, 1997. In addition, two minor amendments to each of claims 38 and 39 correct claim depedency errors and wording. The need for these changes was brought to appellants' attention by the examiner after filing of the first brief. Entry of these amendments also is respectfully requested.

V. SUMMARY OF THE INVENTION

The present invention is drawn to the use of viruses, in combination with radiotherapy, to potentiate the therapeutic effect of each of these agents. Specification at page 4, lines 15-16. In

particular, it has been determined that adenoviruses or herpes simplex viruses act in concert with ionizing radiation to effect enhanced killing in cells treated with both of these agents, as compared with either of these treatments alone. *Id.* at lines 16-19. This aspect of the invention may be employed effectively in the treatment of cancer, specifically, in the inhibition of tumor cell formation and proliferation. *Id.* at lines 19-22.

VI. ISSUE PRESENTED

Are claims 8, 10, 11, 13, 15, 18-27 and 35-55 enabled?

VII. GROUPING OF THE CLAIMS

The claims do not stand or fall together as set forth in §IX, below. In particular, certain dependent claims set forth the very features that are argued by the examiner to be enabling. For example, claims 15 and 24 are separately patentable over the recitation of HSV-1, while claims 51-53 are separately patentable over the recitation of Ad5. Claims 10, 11, 21, 27, 36, 37, 44 and 45 all either recite brain or breast cancer as the target, providing grounds for separate consideration of enablement. Claims 25 and 47 both set forth direct injection of a tumor, thereby establishing a basis for separate patentability.

VIII. SUMMARY OF THE ARGUMENT

According to the examiner, the claims all lack enabling support in the specification and, thus, are rejected under §112, first paragraph. Primarily, the examiner's arguments are directed at the scope of the claims, with particular interest in the types of vectors, the types of tumors to be treated and the mode of administration. However, it is appellants' position that the examiner has not advanced a

credible reason to doubt that the full range of claimed vectors (HSV-1 and HSV-2; Ad5) could be used. Rather, the examiner simply notes that the specification provides limited examples. This cannot serve to shift the burden to appellants, especially in light of additional considerations, presented by appellants, that argue against the examiner's position.

Similarly, the examiner attacks, without support, the treatment of various tumor types. However, it goes unchallenged on the record that adenovirus and herpesvirus can infect a large number of host cells and that radiation has been used successful to treat a plethora of different cancers. Thus, even if the examiner had raised a reasonble *prima facie* case against the claims, these facts would militate against the rejection.

Finally, with respect to routes, it again is submitted that the claims restrict themselves to situations where the delivery is effective, thereby obviating any concerns that the claims encompass inoperative species. Moreover, that is nothing on the record to indicate that other modes of administration, such as local or regional delivery, injection into the tumor's supporting vasculature, or administration to a natural or artificial body cavity, would not work. Thus, despite the fact that the examiner has not offered a reason sufficient to shift the burden to appellants, the examiner's position nonetheless has been rebutted.

IX. ARGUMENT

In the Advisory Action, the examiner failed to mention the rejections under 35 U.S.C. §112, second paragraph. Given that amendments were offered addressing both of the minor

clarity issues raised in the Final Office Action, appellants believe that these rejections have been withdrawn. Thus, the single remaining issue appears to be whether the claims are sufficiently enabled to meet the requirements of 35 U.S.C. §112, first paragraph. The examiner believes they are not, arguing that the specification does not provide examples that support the breadth of the claims with respect to (a) unspecified viral vectors, (b) tumor type and (c) mode of administration. Each of these points is addressed below.

First, the examiner has maintained the objection in as much as the claims are not restricted to HSV-1 and adenovirus type 5. The rationale is as follows:

> ... [I]n the absence of data regarding the specific features of herpesviruses in general, and of HSV viruses in particular, which mediate the inhibition of glioblastoma tumor cell proliferation, it is unpredictable and uncertain whether any virus other than HSV-1 will have the anti-glioblastoma tumor activity of HSV-1 as disclosed in Example 1. Similarly, claims 28 and 34-50 [*sic*, 35-50, 54 and 55] recite any type of adenovirus, yet one skilled in the art would recognize that the different types of adenoviruses also differ concretely in their structures and activities ....

Final Office Action at pages 3-4. Given that the claims have been amended to recite "herpes simplex virus," the entire argument is premised on the fact that HSV-1 differ structurally and functionally from HSV-2, and that adenoviral serotypes similarly differ from one another. No other facts or scientific reasoning are provided, and no explanation of which allegedly distinct structural or functional features of HSV-1 and Ad5 are responsible for the "successful" treatment is provided.

As stated in the previous response, the similarities between HSV-1 and HSV-2 overwhelm the differences, both from structural and functional standpoints. For example, both viruses have double-

stranded DNA genomes of approximately 152 kB, exhibiting a G+C content of 67% (HSV-1) and 69% (HSV-2), and an overall homology of about 80%. They both exhibit "unique long" and "unique short" regions, bounded by inverted terminal repeats. Furthermore, almost every HSV-1 polypeptide has a homolog in HSV-2, with a conservation of 60-80% of amino acid residues. Both being members of the subfamily *Alphaherpesviridae*, HSV-1 and -2 exhibit a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected host cells, and the capacity to establish latent infections primarily, but not exclusively, in sensory ganglia. See Chapters 33-34 by Bernard Roizman in *FUNDAMENTAL VIROLOGY*, 2nd Edition, Fields *et al.* (ed.) (1991) (Exhibit A).

Given these striking similarities, it simply is not true that the skilled artisan would believe that the effects observed with HSV-1 would vary significantly with HSV-2. The only "rationale" offered by the examiner is that "specific features" are not set forth and, therefore, it is unclear whether viruses other than HSV-1 would have these effects. However, a *prima facie* case of nonenablement requires more that mere assertions of "unpredictability." Otherwise, the admonition of the CCPA in *In re Marzocchi*, 169 USPQ 370 (CCPA 1971), that the Patent Office ***must*** take appellants' specification as in compliance with enablement requirements unless there is reason to doubt "the objective truth" of the specification, is meaningless. Simply put, it is not up to appellants' to prove enablement in the first instance, it is up to the examiner to provide reasons to doubt enablement. At least with respect to the claims currently pending, this has not been done. Thus, it is submitted that the record remains devoid of any reason why one would suspect that these two herpes simplex viruses would ***not*** provide the same effects when used in conjunction with radiotherapy.

As for adenoviruses, there similarly is no offered basis for a belief that differing the serotype of adenovirus from type 5, as exemplified, to any of the other 41 serotypes, would cause a different result when practicing the present invention. Rather, the examiner merely has alleged that the various serotypes differ in structure and activity to the point where predictability is lost. Again, this kind of unsupported allegation cannot suffice to shift the burden to appellants to ***prove*** the enabling quality of their disclosure. *In re Marzocchi*. The examiner has, therefore, failed to make out a *prima facie* case of non-enablement here as well.

It should be pointed out, however, that even if the rejection were to be maintained, dependent claims are directed to HSV-1 and Ad5 (claims 15, 24 and 51-53) the subject matter of which has been indicated as outside this rejection. Thus, these claims advance elements which constitute separate grounds for patentability cells, given that the examiner has indicated such subject matter avoids this aspect of the rejection.

Second, the examiner argues that the claims are excessively broad in reciting treatment of all tumor types. Again, there is no reasoning supplied other than vague allegations of "unpredictability" in the field. Appellants respectfully disagree, at least in the context of the instant application. It must be acknowledged that radiation therapy is ***widely*** exploited in the field of cancer therapy. There is little, if any, restriction on the "enablement" of ionizing radiation when applied to the treatment of human malignancies. Similarly, there is no reason to suspect that adenovirus or herpesvirus will selectively infect and replicate ***only*** those cells presented in the instant examples. Rather, the biological characteristics of these vectors suggest the opposite - that they can infect and replicate in a variety of

different cells, including different cancer cells. The present invention merely includes both of these elements and, without a reason to doubt the facts presented above, the skilled artisan would readily accept the *prima facie* enablement of the antitumor effects of (a) herpesviruses & adenoviruses, (b) ionizing radiation and (c) combinations thereof. Moreover, it is ***more*** than credible that the claimed combinations would function similarly, if not in the same manner on most tumor types.

In this regard, appellants also point out that dependent claims 10, 11, 21, 27, 36, 37, 44 and 49 all recite brain and/or breast tumor cells as the target. These claims provide, therefore, separate grounds for patentability with respect to treatment of specific tumor types cells, given that the examiner has indicated such subject matter avoids this aspect of the rejection.

Third, and finally, the examiner again raises the issue of methods and routes of administration. The examiner maintains the position that the claims should be limited to direct intratumoral injection. Again, appellants note that each of the independent claims recites that the virus is (a) either contacted with the tumor cells or (b) delivered to the tumor or tumor site. Thus, the claims themselves, while not limiting the specific ***manner*** in which this occurs, contain an assurance that the virus reaches its target. This is objective enablement, and all that is required to satisfy §112, first paragraph.

Furthermore, those of skill in the art are more than able to determine, depending on the given type or location of the tumor, the precise mode of administration that best exploits the present invention. For example, some tumors are highly vascularized. Thus, an efficient delivery mechanism would to inject the virus by an intravenous or intraarterial route. Other tumor cells exist in natural

body cavities or in cavities created by tumor resection. Virus may be administered to these bodies cavities, dispensing with the need for intratumoral injection. Adenovirus exhibits natural tropism for liver tissue. Similarly, in the case of herpes simplex virus, retrograde transport of the virus to central nervous system tissue would provide another indirect targeting mode. All of these are credible reasons that direct intratumoral injection is not ***required*** for enablement.

As with the other bases for the rejection, the examiner has provided nothing in the way of evidence that would suggest that any specific route, much less direct intratumoral injection, is critical to this aspect of the present invention, which merely involves the administration of infectious viral particles to tumor cells of a patient. Again, it is incumbent upon the examiner to come forward with more than mere allegations of "unpredictability;" there is no initial burden on appellants to ***prove*** enablement. *In re Marzocchi*.

It should be pointed out that dependent claims 25 and 47 both recite injection of virus into a tumor site. Thus, these claims present separate points of patentability for modes of viral delivery to target cells, given that the examiner has indicated such subject matter avoids this aspect of the rejection.

It is respectfully submitted, therefore, that the foregoing rejection, on all asserted grounds, is improper. Reversal thereof by the Board is respectfully requested.

X. SUMMARY AND CONCLUSION

In light of the foregoing remarks, it is respectfully submitted that the appealed claims are enabled. Therefore, reversal of the rejection under 35 U.S.C. §112, first paragraph by the Board is respectfully requested.

Respectfully submitted,

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APPENDIX 1: PENDING CLAIMS

8. The method according to claim 13, wherein the tumor cell is a human tumor cell.

10. The method according to claim 8, wherein the human tumor cell is a brain cancer cell.

11. The method according to claim 8, wherein the human tumor cell is a breast cancer cell.

13. A method of inhibiting growth of a tumor *in vivo* comprising delivering to said tumor, in combination, a herpes simplex virus and ionizing radiation, wherein said combination is sufficient to inhibit the growth of said tumor.

15. The method according to claim 13, wherein the herpes simplex virus is HSV-1.

18. A method of enhancing the effectiveness of ionizing radiotherapy comprising administering to a tumor site in a mammal (i) a pharmaceutical composition comprising a herpes simplex virus and (ii) ionizing radiation, wherein the combination of herpes simplex virus infection and radiation is more effective than ionizing radiation alone.

19. The method according to claim 18, wherein the composition comprises from about 10^8 to about 10^{10} herpesvirus particles.

20. The method according to claim 18, wherein the administering is by means of an oral or intravenous route.

21. The method according to claim 18, wherein the tumor is brain tumor or breast tumor.

22. The method according to claim 18, wherein the mammal is a human.

23. A method of killing a tumor cell comprising the steps of:

(a) contacting said tumor cell with a herpes simplex virus; and
(b) exposing said cell to a dose of ionizing radiation sufficient to kill said cell in conjunction with said herpes simplex virus.

24. The method according to claim 23, wherein the herpes simplex virus is HSV-1.

25. The method according to claim 13, wherein said delivering comprises injecting into a tumor site a pharmaceutical composition comprising said herpes simplex virus.

26. The method according to claim 13, wherein the tumor is exposed to ionizing radiation selected from the group consisting of X-irradiation, γ-irradiation and β-irradiation.

27. The method according to claim 13, wherein the tumor is a brain tumor or a breast tumor.

35. The method according to claim 46, wherein the tumor cell is a human tumor cell.

36. The method according to claim 35, wherein the human tumor cell is a brain cancer cell.

37. The method according to claim 35, wherein the human tumor cell is a breast cancer cell.

38. (Amended) The method according to claim [34] 46, wherein tumor the cell is located within an animal, and the adenovirus is administered to the animal in a pharmaceutically acceptable form.

39. (Amended) The method according to claim [34] 46, wherein the tumor cell is exposed to X-irradiation, γ-irradiation, or β-irradiation.

40. A method of inhibiting growth of a tumor *in vivo* comprising delivering to said tumor, in combination, an adenovirus lacking an exogenous therapeutic gene and ionizing radiation, wherein said combination is sufficient to inhibit the growth of said tumor.

41. A method of enhancing the effectiveness of ionizing radiotherapy comprising administering to a tumor site in a mammal (i) a pharmaceutical composition comprising a adenovirus lacking an exogenous therapeutic gene and (ii) ionizing radiation, wherein the combination of adenovirus infection and radiation is more effective than ionizing radiation alone.

42. The method according to claim 41, wherein the composition comprises from about 10^8 to about 10^{11} adenovirus particles.

43. The method according to claim 41, wherein the tumor is exposed to ionizing radiation selected from the group consisting of X-irradiation, γ-irradiation and β-irradiation.

44. The method according to claim 41, wherein the tumor is brain tumor or breast tumor.

45. The method according to claim 41, wherein the mammal is a human.

46. A method of killing a tumor cell comprising the steps of:

a) contacting said tumor cell with an adenovirus lacking an exogenous therapeutic gene; and

b) exposing said cell to a dose of ionizing radiation sufficient to kill said cell in conjunction with said adenovirus.

47. The method according to claim 46, wherein said delivering comprises injecting into a tumor site a pharmaceutical composition comprising said adenovirus.

48. The method according to claim 46, wherein the tumor is exposed to ionizing radiation selected from the group consisting of X-irradiation, γ-irradiation and β-irradiation.

49. The method according to claim 46, wherein the tumor cell is a brain tumor cell or a breast tumor cell.

50. The method according to claim 46, wherein the composition comprises from about 10^8 to about 10^{11} adenovirus particles.

51. The method of claim 40, wherein said adenovirus is Ad5.

52. The method of claim 41, wherein said adenovirus is Ad5.

53. The method of claim 46, wherein said adenovirus is Ad5.

54. The method of claim 41, wherein said composition is administered intravenously.

55. The method of claim 55, wherein said composition comprises from about 10^8 to about 10^{11} adenovirus particles.

SECOND EDITION

FUNDAMENTAL VIROLOGY

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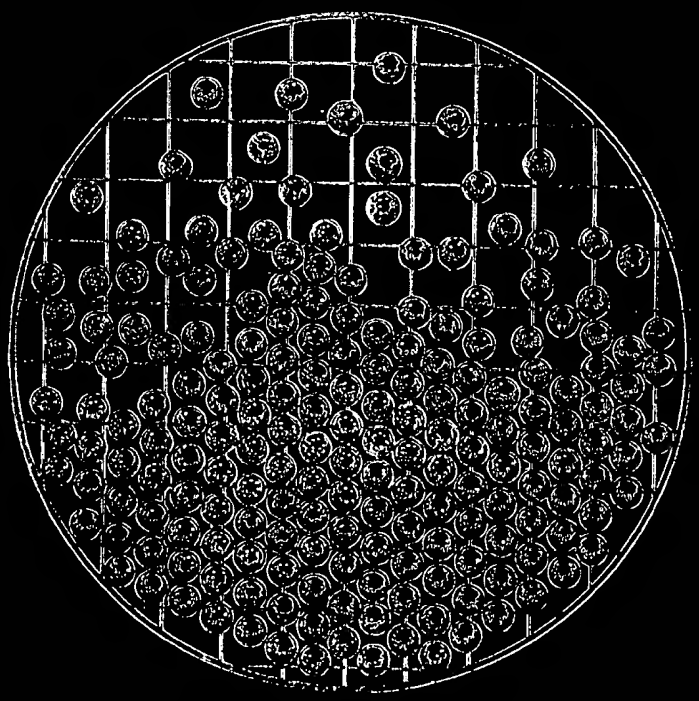

RAVEN PRESS

Fundamental Virology, Second Edition, edited by B. N. Fields, D. M. Knipe et al.
Raven Press, Ltd., New York © 1991.

CHAPTER 33

Herpesviridae: A Brief Introduction

Bernard Roizman

Who made the world I cannot tell;
'Tis made, and here I am in hell.
My hand, though now my knuckles bleed,
I never soiled with such a deed.
A. E. HOUSMAN, No. XIX in *More Poems*

DEFINITION

Inclusion in the Family Herpesviridae

Membership in the family Herpesviridae is based on the architecture of the virion (Fig. 1). A typical herpesvirion consists of (a) a core containing a linear, double-strand DNA, (b) an icosadeltahedral capsid, approximately 100–110 nm in diameter, containing 162 capsomeres with a hole running down the long axis, (c) an amorphous, sometime asymmetric material that surrounds the capsid, designated as the *tegument*, and (d) an envelope containing viral glycoprotein spikes on its surface.

Distribution in Nature

Herpesviruses are highly disseminated in nature, and most animal species have yielded at least one herpesvirus upon examination. Of nearly 100 herpesviruses that have been at least partially characterized, six herpesviruses have been isolated so far from humans [herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein–Barr virus (EBV), and human herpesvirus 6 (HHV6)], four from horses, at least three from cattle, two from pigs [pseudorabies virus (PSV) and porcine cytomegalovirus], and three from chickens [infectious laryngotracheitis virus and two viruses associated with Marek's disease]. Representatives of some of the better known viruses are listed in Table 1.

B. Roizman: The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637.

ARCHITECTURE

Structural Components

The Core

The core of the mature virion contains the viral DNA in the form of a torus (26,49). In some herpesvirions, the torus appears to be suspended by a proteinaceous spindle consisting of fibrils embedded in the underside of the capsid and passing through the hole of the torus. The precise arrangement of the DNA in the toroid is not known.

The Capsid

The structural features of the capsid (i.e., its 100-nm diameter and 162 capsomeres) are characteristic of all herpesviruses. The pentameric capsomeres at the vertices have not been well characterized. The hex-

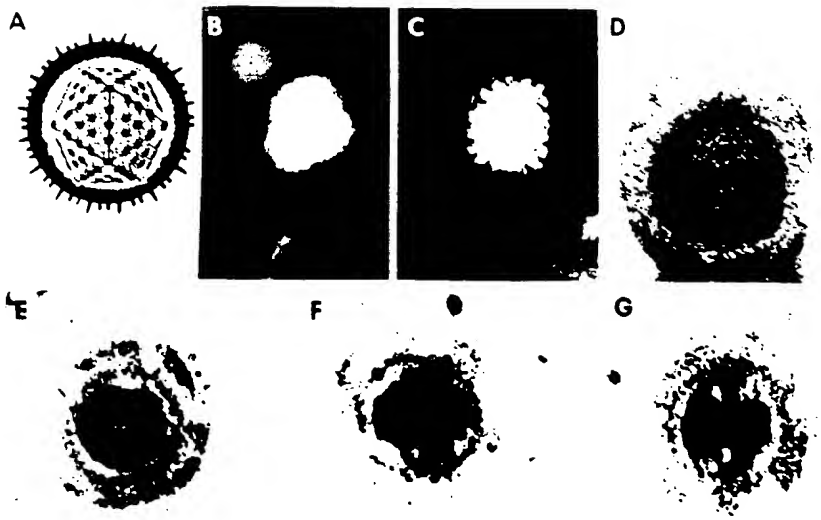

FIG. 1. The morphology of herpesviruses. **A**: Schematic representation of the herpesvirion seen through a cross section of the envelope with spikes projecting from its surface. The sides of the icosadeltahedron forming the capsid show a twofold symmetry. The irregular inner perimeter of the envelope is meant to represent the occasional asymmetric arrangement of the tegument. **B**: An intact, negatively stained HSV-1 virion. The intact envelope is not permeable to negative stain. The diameter of the virion is approximately 120 nm. **C**: HSV-1 capsid exposed to negative stain and showing twofold symmetry matching the diagrammatic representation of the capsid in panel A. **D**: HSV-1 capsid containing DNA permeated with uranyl acetate. The electron micrograph shows the presence of thread-like structures 4–5 nm wide on the surface of the core. **E, F,** and **G**: Electron micrographs of thin sections of HSV-1 virions showing the core cut at different angles. The preparation was stained with uranyl acetate and counterstained with lead citrate. The DNA core preferentially takes up the stain and appears as a toroid with an outer diameter of 70 nm and an inner diameter of 18 nm. The toroid appears to be suspended by a fibrous cylindrical structure. The micrographs show the toroid seen looking down the hole (panel E), in cross section (panel F), or from the side (panel G). (Panels D–G are from ref. 26.)

americ capsomeres are 9.5 × 12.5 nm in longitudinal section; a channel that is 4 nm in diameter runs part way from the surface along the long axis (66).

The Tegument

The *tegument*, a term introduced by Roizman and Furlong (55) to describe the structures between the capsid and envelope, has no distinctive features in thin sections, but it may appear to be fibrous on negative staining (47,48,66). The thickness of tegument may vary, depending on the location of the virion within the infected cell; when the amount is variable, there is more of it in virions accumulating in cytoplasmic vacuoles than in those accumulating in the perinuclear space (25). The available evidence suggests that the amount of tegument is more likely to be determined by the virus than by the host (44). The tegument is frequently distributed asymmetrically.

The Envelope

Electron-microscopic studies on thin sections have shown that the outer covering, the *envelope*, of the virus has a typical trilaminar appearance (20); the envelope appears to be derived from patches of altered cellular membranes (1,23,48). The presence of lipids was demonstrated by analyses of virions (2,5) and by the sensitivity of the virions to lipid solvents and detergents (33,61,62). The herpesvirus envelope contains numerous protrusions of spikes, which are more numerous and shorter than those appearing on the surface of many other enveloped viruses. Wildy and Watson (66) estimated that the spikes on HSV virions are approximately 8 nm long. The spikes consist of glycoproteins (63). The number and relative amounts of viral glycoproteins vary; HSV specifies at least eight glycoproteins (see Chapter 34).

The Virion

The size of herpesvirions has been reported to vary from 120 nm to nearly 300 nm (reviewed in ref. 55). The variation is, in part, due to variability in the thickness of tegument. Another major source of variability is the state of the envelope. Intact envelopes are impermeable and generally retain the quasi-spherical shape of the virion. Damaged envelopes are permeable to negative stains; permeated virions have a sunny-side-up egg appearance, with a diameter much larger than that of an intact virion.

The precise number of polypeptide species contained in the herpesvirions is not known and may vary from one virus to another. The estimates generally range from 30 to 35 polypeptides.

TABLE 1. *Selected viruses of the family Herpesviridae*

Designation	Common Name (synonyms)	Subfamily	G + C (mole %)	Genome properties; class and $M_r \times 10^6$ (or kilobase pairs)[a]	References
Viruses of humans					
Human herpesvirus 1	Herpes simplex virus 1	α	67	E(152)	31,45,53
Human herpesvirus 2	Herpes simplex virus 2	α	69	E(152)	53,58
Human herpesvirus 3	Varicella-zoster virus	α	46	D(125)	16,19,41
Human herpesvirus 4	Epstein–Barr virus	γ	60	C(172)	3,21
Human herpesvirus 5	Cytomegalovirus	β	57	E(229)	60
Human herpesvirus 6			42[b]	A(170)[b]	40,57
Viruses of nonhuman primates					
Aotine herpesvirus 1	Herpesvirus aotus type 1	β	56	E;145	4,13
Aotine herpesvirus 2	Herpesvirus aotus type 2	γ	—	B;100	4
Ateline herpesvirus 2	Herpesvirus aotus type 3	β	56	E;145	13,14
	Herpesvirus ateles strain 810	γ	48	B;90	24,46
Cercopithecine herpesvirus 1	B virus	α	75	E;107	56
Cercopithecine herpesvirus 2	SA8	α	67	E;100	30
Cercopithecine herpesvirus 12	Herpesvirus papio; baboon herpesvirus	γ	—	C;110	22
Pongine herpesvirus 1	Chimpanzee herpesvirus; herpesvirus pan	γ	—	C;110	34,38
Saimirine herpesvirus	Squirrel monkey virus; herpesvirus saimiri	γ	46	B;103	46,52
Viruses of other mammals					
Bovine herpesvirus 1	Infectious bovine rhinotracheitis virus	α	72	D	27,42
Bovine herpesvirus 2	Bovine mammillitis virus	α	64	E;88	43,64
Equid herpesvirus 1	Equine abortion virus	α	57	D;94	52
Suid herpesvirus 1	Pseudorabies virus	α	74	D;91	6,32
Viruses of birds					
Gallid herpesvirus 2	Marek's disease herpesvirus 1	γ	46	E;110	11,39
Gallid herpesvirus 3	Marek's disease herpesvirus 2				9,59
Meleagrid herpesvirus 1	Turkey herpesvirus	γ	48	E;104	36,39
Viruses of fish					
Ictalurid herpesvirus 1	Channel catfish virus (CCV)	α	56	A;86	12,67

[a] Kilobase-pair values appear inside parentheses; molecular-weight values are preceded by a semicolon.
[b] P. E. Pellett, *personal communication.*

Herpesvirus DNAs

Size, Conformation, and Base Composition

The herpesvirus DNAs extracted from virions and characterized to date are linear and double-stranded, but they circularize immediately upon release from capsids into the nuclei of infected cells.

The variable features of the herpesvirus DNAs are their molecular weight and base composition. The molecular weight of herpesvirus DNAs varies from approximately 80 to 150 million, or the genomes range from approximately 120 to 230 kilobase pairs (Table 1). The variability in the size of herpesvirus DNAs does not reflect a polymorphism in the size of DNAs of individual viruses. The variation in the size of the genome of any one herpesvirus appears to be minimal, but not insignificant. Thus, many viral DNAs contain terminal and internal reiterated sequences. Because of variability in the number of these reiterations, the size of individual genomes may vary by >10 kilobase pairs. Spontaneous deletions also occur; they have been noted in both HSV and EBV strains passaged outside the human host (e.g., EBV strain P3HR1, HSV strain HFEM).

The base composition of herpesvirus DNAs varies from 31 to 75 G+C moles percent (Table 1). Furthermore, herpesvirus DNAs vary with respect to the extent of homogeneity of base sequence distribution across the length of the genome. The extent of inho-

mogeneity in the base composition varies from minimal (e.g., HSV) to very extensive (e.g., the DNAs of herpes saimiri and herpes ateles; see ref. 7).

Sequence Arrangements in Herpesvirus DNAs

Probably the most interesting feature of herpesvirus DNAs is their sequence arrangement. The sequence arrangement shown in Fig. 2 emphasizes the presence and location of reiterations of terminal sequences greater than 100 base pairs. According to this scheme, the herpesviruses can be divided into six groups designated by the letter A to F. In the genomes of viruses comprising group A and exemplified by the channel catfish herpesvirus, a large sequence from one terminus is directly repeated at the other terminus. In the group B genomes exemplified by herpes saimiri virus, the terminal sequence is directly repeated numerous times at both termini; furthermore, the number of reiterations at both termini may vary. In the group C genomes exemplified by EBV, the number of direct terminal reiterations is smaller; however, there may be other, unrelated sequences greater than 100 base pairs which are directly repeated and which subdivide the unique (or quasi-unique) sequences of genome into several well-delineated stretches. In group D genomes exemplified by that of VZV, the sequences from one terminus is repeated in an inverted orientation internally. In these genomes, the domain consisting of the stretch of unique sequences flanked by inverted repeats [small (S) component] can invert relative to the remaining sequences [large (L) component] such that the DNA extracted from virions or infected cells consists of two equimolar populations differing solely in the relative orientation of the S component relative to the L component. In group E viral genomes exemplified by those of HSV and HCMV, sequences from both termini are repeated in an inverted orientation and juxtaposed internally, dividing the genomes into two components, each of which consists of unique sequences flanked by inverted repeats. In this instance, both components can invert relative to each other, and DNA extracted from virions or infected cells consists of four equimolar populations differing in the relative orientation of the two components. In the genomes comprising the F group exemplified by that of the tupaia herpesvirus, the sequences at the two termini are not identical and are not repeated directly or in an inverted orientation.

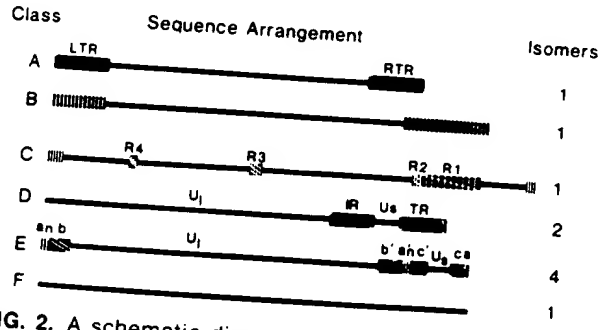

FIG. 2. A schematic diagram of the sequence arrangements in the six classes of genomes of the viruses comprising the family Herpesviridae. The genomes A, B, C, D, E, and F are exemplified by the channel catfish herpesvirus, herpesvirus saimiri, Epstein–Barr virus, varicella-zoster virus, herpes simplex viruses, and tupaia herpesvirus, respectively. In the schematic diagram, the horizontal lines represent unique or quasi-unique regions. The reiterated domains are shown as rectangles and are designated as follows: left and right terminal repeats (LTR and RTR) for class A; repeats R1 to R4 for internal repeats of class C; and internal and terminal (IR and TR) repeats of class D. The termini of class E (e.g., HSV) consist of two elements. One terminus contains *n* copies of sequence *a* next to a larger sequence designated as *b*. The other terminus has one directly repeated *a* sequence next to a sequence designated as *c*. The terminal *ab* and *ca* sequences are inserted in an inverted orientation (denoted by primes), separating the unique sequences into long (U_L) and short (U_S) domains. Terminal reiterations in the genomes of class F have not been described. In class B, the terminal sequences are reiterated numerous times at both termini. The number of reiterations at each terminus may vary. The components of the genomes in classes D and E invert. In class D, the short component inverts relative to the long. Although the long component may also invert (rarely), most of the DNA forms two populations differing in the orientation of the short component. In the class E genomes, both the short and long components can invert, and viral DNA consists of four equimolar isomers.

BIOLOGICAL PROPERTIES

The known herpesviruses appear to share four significant biological properties:

1. All herpesviruses specify a large array of enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase, etc.), DNA synthesis (e.g., DNA polymerase helicase, primase), and, possibly, processing of proteins (e.g., protein kinase), although the exact array of enzymes may vary somewhat from one herpesvirus to another (see Chapters 34–36, and References 69–71).
2. Both the synthesis of viral DNAs and the assembly of capsids occur in the nucleus. In the case of some herpesviruses, it has been claimed that the virus may be de-enveloped and re-enveloped as it transits through the cytoplasm. Irrespective of the merits of these conclusions, envelopment of the capsids as it transits through the nuclear membrane is obligatory.
3. Production of infectious progeny virus is invariably accompanied by the irreversible destruction of the infected cell.

4. The herpesviruses examined to date are able to remain latent in their natural hosts. In cells harboring latent virus, the viral genomes take the form of closed circular molecules, and only a small subset of viral genes is expressed.

Herpesviruses also vary greatly in their biologic properties. Some have a wide host-cell range, multiply efficiently, and rapidly destroy the cells that they infect (e.g., HSV-1, HSV-2, etc.). Others (e.g., EBV, HHV6) have a narrow host-cell range. The multiplication of some herpesviruses (e.g., HCMV) appears to be slow. While all herpesviruses remain latent in a specific set of cells, the exact cell in which they remain latent varies from one virus to another. For example, whereas latent HSV is recovered from sensory neurons, latent EBV is recovered from B lymphocytes. Herpesviruses differ with respect to the clinical manifestations of diseases they cause.

CLASSIFICATION

Current Classification

The purpose of classifying viruses into subfamilies and genera is multifold. While a classification scheme is often used to depict evolutionary relatedness, it also serves a practical purpose of enabling the laboratory worker to predict the properties and identity of a new isolate. The members of the family Herpesviridae have been classified by the Herpesvirus Study Group of the International Committee on the Taxonomy of Viruses (ICTV) into three subfamilies—the Alphaherpesvirinae, the Betaherpesvirinae, and the Gammaherpesvirinae—on the basis of biologic properties (54). The same study group classified a small number of herpesviruses into genera based on DNA sequence homology, similarities in genome sequence arrangement, and relatedness of important viral proteins demonstrable by immunologic methods. While a few genes [e.g., homologues of glycoprotein B of HSV-1 (see refs. 51 and 65) or of glycoprotein H (see ref. 29)] are conserved among members of different subfamilies, nucleic acid and protein sequence homologies are particularly useful for the classification of viruses that are closely related (genera).

A formal binomial nomenclature is not currently applied. The recommendations of the study group endorsed by ICTV is that herpesviruses be designated by serial arabic number and the family (most cases) or subfamily (for primates and some animals) in which the natural host of the virus is classified (e.g., human herpesvirus 6, circopithecine herpesvirus 1, etc.; see Table 1).

Alphaherpesvirinae

The members of this subfamily are classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily, but not exclusively, in sensory ganglia. This subfamily contains the genera *Simplexvirus* (HSV-1, HSV-2, circopithecine herpesvirus 1, bovine mamillitis virus) and *Varicellovirus* (VZV, pseudorabies virus, and equine herpesvirus 1).

Betaherpesvirinae

A nonexclusive characteristic of the members of this subfamily is a restricted host range. The reproductive cycle is long, and the infection progresses slowly in culture. The infected cells frequently become enlarged (cytomegalia), and carrier cultures are readily established. The virus can be maintained in latent form in secretory glands, lymphoreticular cells, kidneys, and other tissues. This subfamily contains the genera *Cytomegalovirus* (HCMV) and *Muromegalovirus* (murine cytomegalovirus).

Gammaherpesvirinae

The experimental host range of the members of this subfamily is limited to the family or order to which the natural host belongs. *In vitro*, all members replicate in lymphoblastoid cells, and some also cause lytic infections in some types of epithelioid and fibroblastic cells. Viruses in this group are specific for either T or B lymphocytes. In the lymphocyte, infection is frequently either at a pre-lytic or lytic stage, but without production of infectious progeny. Latent virus is frequently demonstrated in lymphoid tissue. This subfamily contains two genera, namely, *Lymphocryptovirus* (e.g., EBV), and *Rhadinovirus* (herpesvirus ateles and herpesvirus saimiri).

Future Trends in Herpesvirus Classification

The current classification of herpesviruses can be described as being simple, fortuitously appropriate, and defective. Its defect is that it is not based on objective criteria that serve a useful function in defining evolutionary relatedness. The objective criteria that are available or have been proposed for the classification of herpesvirus subfamilies are as follows: (i) conservation of genes and gene clusters (e.g., DNA polymerase, glycoproteins B, C, and H, single-strand DNA-binding protein, major capsid protein, etc.) and arrangement of gene clusters relative to each other; (ii)

the arrangement of the terminal sequences involved in packaging of the viral genome; and (iii) the presence and distribution of nucleotides that are subject to methylation (10,15,17,18,28,35,37,50). Fortuitously, most herpesviruses assigned to the three subfamilies would have been assigned to these subfamilies by most of the objective criteria. The major exceptions are the Marek's disease herpesviruses, which share biological properties with members of the Gammaherpesvirinae but which, by some objective criteria (conservation and collinearity of gene clusters), end up among the Alphaherpesvirinae (8). HHV6 is another example of a virus which, by biologic criteria, should be classified into the Gammaherpesvirinae but which, by objective criteria enumerated above, is classified among the Betaherpesvirinae.

The correlation between the biologic and molecular criteria which can be applied today to produce a similar clustering of viruses into subfamilies is gratifying but not necessarily reassuring. The quandary associated with the classification of Marek's disease viruses and of HHV6 are portents of problems yet to come. The emphasis on the known genes and their conservation and relative arrangement, however, may be misplaced. The key issue is that the three subfamilies differ from each other in fundamental biologic properties, and the objective criteria that should be used to describe them should reflect these properties. In the case of Marek's disease virus, the genes conserved and collinear with those of VZV (8) are not the domains expressed during latency or in transformed cells. Delineation and evolutionary relatedness of genes responsible for biologic properties may be a more significant criterion for both evolutionary relatedness and classification than the arrangement and evolution of genes conserved throughout the family Herpesviridae, but these are not yet known. Regardless of the criteria that will be used, evolutionary trees will inevitably grace the herpesvirus texts in the not-too-distant future.

The focus of the chapters in the section dealing with Herpesviridae (Chapters 34–36, and References 68–71) is on the six known human herpesviruses and on herpes B virus, the one nonhuman primate virus known to be able to enter and spread in the human population.

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Fundamental Virology, Second Edition, edited by
B. N. Fields, D. M. Knipe et al.
Raven Press, Ltd., New York © 1991.

CHAPTER 34

Herpes Simplex Viruses and Their Replication

Bernard Roizman and Amy E. Sears

> On fait la science avec des faits, comme on fait une maison avec des pierres; mais une accumulation de faits n'est pas plus une science qu'un tas de pierres n'est une maison.
>
> HENRI POINCARE

Herpes simplex viruses (HSVs) were the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses. Their attractions are their biologic properties and, in particular, their ability to cause a variety of infections, to remain latent in their host for life, and to be reactivated to cause lesions at or near the site of initial infection. They serve as models and tools for the study of translocation of proteins, synaptic connections in the nervous system, membrane structure, gene regulation, and a myriad of other biological problems, both general to viruses and specific to HSV. For years, their size and complexity served as a formidable obstacle to intensive research. More than 40 years passed from the time of their isolation until Schneweiss (444) demonstrated that there were, in fact, two serotypes, HSV-1 and HSV-2, whose formal designations under ICTV

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rules are now human herpesviruses 1 and 2 (416). Not until 1961 were plaque assays published (420), and only much later were the genome sizes and the extent of homology between these two viruses reported. This chapter recounts well-established facts; however, its main emphasis is on burning issues, the problems whose time has come.

Virology conserves two myths. The first is that research on a virus reaches its peak when the number of investigators approaches the number of nucleotides in its genome. This formula calls for 152,000+ investigators, one for each base pair (222,294). In orders of magnitude, we are close but not yet there. There are times when we think almost that many bodies will be needed to unravel all the mysteries of these viruses.

The second myth is that virologists repeat the same experiment over and over again. As in all myths, there may be a grain of truth here. In wading through the mass of articles published in the past few years, it was instructive to see how many times the same phenomenon was published or rediscovered time and time again. This apparently is not only a reflection of George Santayana's injunction that "Those who cannot remember the past are condemned to repeat it" but is also because only the last reported experiment is remembered, correct or not. We have taken pains to correct the record.

VIRION STRUCTURE

As with all herpes virions, the HSV virion consists of four elements: (i) an electron-opaque core, (ii) an icosadeltahedral capsid surrounding the core, (iii) an amorphous tegument surrounding the capsid, and (iv) an outer envelope exhibiting spikes on its surface.

Virion Polypeptides

Studies on purified HSV-1 virions indicated that they contain approximately, but probably not less than, 33 proteins designated as virion polypeptides (VPs) and given serial numbers (170,478). All of the virion proteins were made after infection, and no host protein could be detected in purified preparations. Of the approximately 33 proteins, eight are on the surface of the virion and are glycosylated. These glycoproteins are gB (VP7 and VP8.5), gC (VP8), gD (VP17 and VP18), gE (VP12.3 and VP12.6), gH/gG, and gI. Another gH small glycoprotein, which will be given the designation gJ, predicted by DNA sequence analyses, has recently been demonstrated by N. Frenkel and associates (N. Frenkel, *personal communication*). Some of the surface glycoproteins have been shown to be components of the envelope spikes (482).

Gibson and Roizman (148,150) described three kinds of capsids, namely, those that lack DNA and were never enveloped (type A), those that contain DNA and were never enveloped (type B), and those that contain DNA and were obtained by de-enveloping intact virions (type C). The empty capsids consist of five proteins, namely, VP5, VP19C, VP23, VP24, and a smaller (12,000 molecular weight) protein described subsequently (64). VP5 was estimated to be present in ratios of 850–1,000 per virion [i.e., approximately six per hexameric capsomere (170,417,511)], but recent studies (445) suggest that VP5 is a component of both pentameric and hexameric capsomeres. VP19C and VP5 appear to be linked by disulfide bond (550) and are present in approximately similar ratios per virion (170). Braun et al. (32) showed that VP19C, identified as the infected cell protein (ICP) 32, bound DNA and was probably involved in anchoring the viral DNA in the capsid. Recent studies by Sherman and Bachenheimer (462) suggest that the type A capsids are not in the pathway of virion maturation and may be a decay product.

Type B capsids differ from the A type in that they contain two additional proteins, namely, VP21 and VP22a. Type C capsids were reported to contain a smaller protein VP22 but not VP22a (148,150). VP22 and VP22a have similar characteristics (148,150) and were thought to be related constituents of the ICP35 family of proteins present on the surface of the capsid (34) and required for encapsidation of viral DNA (377,399,462). Gibson and Roizman (148) suggested that VP21 is an internal capsid protein.

Depending on the procedure for stripping the envelope, the type C capsids may contain variable amounts of tegument proteins. The type C capsids lack protein VP22a but contain protein VP22. Gibson and Roizman (148,150) concluded that VP22 is processed from VP22a by proteolytic cleavage, since the proteins appear to have similar characteristics. Recent studies by Sherman and Bachenheimer (462) and Rixon et al. (399) suggest that the VP22 found in the type C capsids may not be related to VP22a.

The space between the undersurface of the envelope and the surface of the capsid was designated as the *tegument* (417); it contains the rest of the virion proteins. The most notable of the proteins associated with the underside of the envelope and the capsid are the α-*trans*-inducing factor (αTIF; ICP25; VP16), the virion host shut-off (VHS) protein, and a very large protein (VP1) associated with a complex that binds to the terminal (*a*) sequence of the viral genome. Extensive discussion of the various types of capsids and virions was published by Roizman and Furlong (417). Schrag et al. (445) have reported an elegant model of the HSV-1 capsid.

Viral DNA

Like other herpesvirus DNAs, HSV DNA is linear and double-stranded (23,222,361). In the virion, HSV DNA is packgaged in the form of a toroid (141). The ends of the genome are probably held together or are in close proximity inasmuch as the DNA circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells (362). DNA extracted from virions contains nicks and gaps (27,135,540).

The HSV genome is approximately 150 kilobase pairs, with a G+C content of 68% (HSV-1) or 69% (HSV-2) (23,222,294). It consists of two covalently linked components, designated as L (long) and S (short) (Fig. 1). Each component consists of unique sequences bracketed by inverted repeats (460,514). The repeats of the L component are designated *ab* and *a'b'*, while those of the S component are designated *a'c'* and *ca* (514) (Fig. 1). The number of *a* sequence repeats at the L–S junction and at the L terminus is variable; the HSV genome can then be represented as

$$a_L a_n b\text{—}U_L\text{—}b'a'_m c'\text{—}U_s\text{—}ca_s$$

where a_L and a_s are terminal sequences with unique properties described below, and a_n and a_m are terminal *a* sequences directly repeated zero or more times (n) or present in one to many copies (m) (87,270,411,412, 504,514,519). The structure of the *a* sequence is highly conserved but consists of a variable number of repeat elements. In the HSV-1(F) strain, the *a* sequence consists of a 20-base-pair direct repeat (DR1), a 65-base-pair unique sequence (U_b), a 12-base-pair direct repeat (DR2) present in 19–23 copies per *a* sequence, a 37-base-pair direct repeat (DR4) present in two to three copies, a 58-base-pair unique sequence (U_c), and a final copy of DR1 (318,320). The size of the *a* sequence varies from strain to strain, reflecting in part the number of copies of DR2 and DR4. The structure of the *a* sequence can be represented as

$$DR1\text{—}U_b\text{—}DR2_n\text{—}DR4_m\text{—}U_c\text{—}DR1$$

with adjacent *a* sequences sharing the intervening DR1. Linear virion DNA contains asymmetric ends, with the terminal *a* sequence of the L component (a_L) ending with 18 base pairs and one 3′ nucleotide extension, and the terminal *a* sequence of the S component (a_s) ending with a DR1 containing only 1 base pair and one 3′ overhanging nucleotide (320).

The L and S components of HSV can invert relative to one another, yielding four linear isomers (Fig. 1) (92,169). The isomers have been designated as P (prototype), I_L (inversion of the L component), I_s (inversion of the S component), and I_{SL} (inversion of both S and L components) (169,323,324).

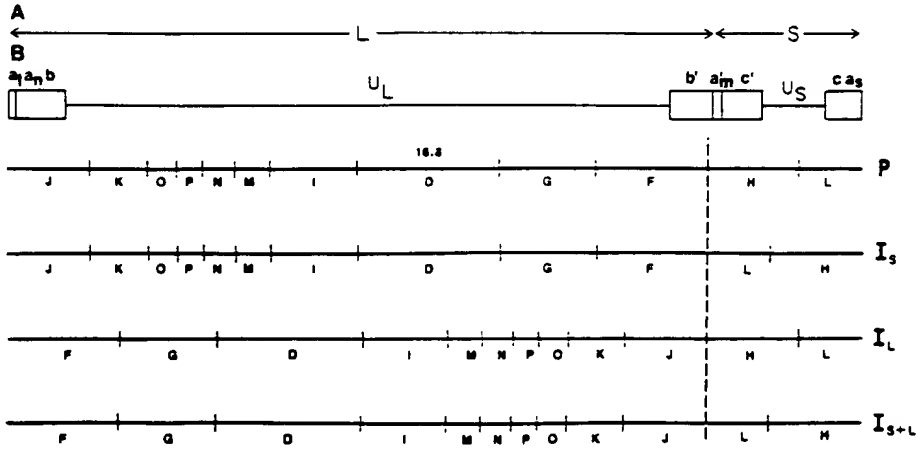

FIG. 1. Schematic representation of the arrangement of DNA sequences in the HSV genome. **A:** The domains of the L and S components are denoted by the arrows. The second line shows the unique sequences (*thin lines*) flanked by the inverted repeats (*boxes*). The letters above the second line designate the following: the terminal *a* sequence of the L component (a_L); a variable (n) number of additional *a* sequences; the *b* sequence; the unique sequence of the L component (U_L); the repetition of the *b* and of a variable (m) number of *a* sequences (a_m); the inverted *c* sequence (c'); the unique sequence of the S component (U_s); and, finally, the terminal *a* sequence (a_s) of the S component. **B:** The Bg1II restriction endonuclease map of HSV-1(F) strain for the P, I_s, I_L, and I_{SL} isomers of the DNA. Note that because Bg1II does not cleave within the inverted repeat sequences, there are four terminal fragments and four fragments spanning the internal inverted repeats in concentrations of 0.5 and 0.25 M, respectively, relative to the concentration of the viral DNA.

The evidence for the repetition of terminal sequences in inverted orientation was based on electron-microscopic studies of denatured HSV-1 DNA allowed to self-anneal (460). These studies, as well as partial denaturation profiles of HSV DNA, revealed that the terminal repeats are repeated internally and that the repeats of each end differ in size and sequence arrangements (92,514). The demonstration that restriction endonucleases which cleave outside inverted repeats yield four 0.5 M terminal fragments and four 0.25 M L–S component junction fragments (169) supported the conclusion that L and S components can invert relative to each other.

The internal inverted repeat sequences are not essential for growth of the virus in cell culture; mutants from which portions of unique sequences and most of the internal inverted repeats have been deleted have been obtained in all four arrangements of HSV DNA (203,363). The genomes of these mutants do not invert; each is frozen in one arrangement of the L and S components, but all retain their viability in cell culture.

Other Constituents

Polyamines

The search for polyamines in the virion evolved from the observations that HSV capsid assembly requires addition of arginine to the medium (284,426,498) and that the capsid does not contain highly basic proteins that would neutralize viral DNA for proper folding inside the capsid. Highly purified virions contain the polyamines spermidine and spermine in a nearly constant ratio, yielding approximately 70,000 molecules of spermidine and 40,000 molecules of spermine per virion (147,149). The polyamines appear to be tightly bound, and they cannot be exchanged with exogenously added labeled polyamines. Disruption of the envelope with nonionic detergents and urea removed the spermidine but not the spermine. The spermine contained in the virion is sufficient to neutralize approximately 40% of the DNA phosphate (149). Parenthetically, proteins have been noted in association with the toroidal structure (141) in the capsid, and a capsid protein has been reported to bind DNA (32).

The compartmentalization of spermine and spermidine may reflect the distribution of polyamines in the infected cell. It is of interest to note that after infection, the conversion of ornithine to putrescine appears to be blocked, but the synthesis of spermine and spermidine does not appear to be affected (147).

Lipids

It has been assumed that HSV acquires its envelope lipids from the host cells. Little is known of the composition of the lipids in the envelopes. The hypothesis that it is determined by the host was supported by the observation that the buoyant density of the virus was host-cell-dependent on serial passage of HSV-1 alternately in HEp-2 and chick embryo cells (477). Since the envelope is derived from cellular membranes, it has been assumed that the viral envelope and cellular membranes contain similar or identical lipids. Even when analyses of viral lipids were in vogue, little was learned of the lipids in the HSV virion. What little is known has been reviewed in detail elsewhere (417).

HSV Polymorphism

Intertypic Variation

Although the genetic maps of HSV-1 and HSV-2 are largely collinear, they differ in restriction endonuclease cleavage sites and in the apparent sizes of viral proteins. Thus, the initial locations of viral genes on the linear map of HSV genomes were based on analyses of HSV-1 × HSV-2 recombinants and took advantage of (a) the intertypic difference in the sizes of the proteins and (b) the locations of restriction endonuclease cleavage sites (286,323,324,378).

Intratypic Variation

The first evidence of intratypic polymorphism emerged from studies of virion structural proteins and indicated that nonglycosylated proteins vary sufficiently in electrophoretic mobility to be used as strain markers (355). Intratypic variability was also noted by Pereira et al. (356) in their studies on the distribution of epitopes to specific monoclonal antibodies among HSV-1 and HSV-2 isolates. The usefulness of virion proteins as markers for molecular epidemiologic studies was limited by the effort required to purify virions for such analyses.

At the DNA level, differences between HSV-1 strains appear to result from (a) base substitutions which may add or eliminate a restriction endonuclease cleavage site and which may, on occasion, change an amino acid or (b) variability in the number of repeated sequences present in a number of regions of the genome (e.g., γ34.5, US11, etc.) (57,400). The restriction endonuclease cleavage patterns of a given strain are relatively stable, whereas the number of repeats are not (37,168,415,504). Thus, no changes in restriction endonuclease patterns were noted in isolates from the same individual over an interval of 13 years or in genomes of an HSV-1 strain passaged serially numerous times in cell culture. However, restriction endonuclease site polymorphism was readily noted in isolates from epidemiologically unrelated individuals

(164,427). On the basis of these properties, restriction endonuclease site polymorphism was used in several epidemiologic studies of HSV transmission in the human population (37,415,427), and restriction endonuclease analyses of coded virus isolates have been used to trace the spread of infection from patients to hospital personnel (35), from patient to patient (268), and from hospital personnel to patient (4,36).

VIRAL REPLICATION

The General Pattern of Replication

It is convenient to begin this section on viral replication with a bird's-eye view of the major events (Fig. 2).

To initiate infection, the virus must attach to cell receptors. Fusion of the envelope with the plasma membrane rapidly follows the initial attachment. The de-enveloped capsid is then transported to the nuclear pores, where DNA is released into the nucleus.

Transcription, replication of viral DNA, and assembly of new capsids take place in the nucleus (Fig. 3).

Viral DNA is transcribed throughout the reproductive cycle by host RNA polymerase II, but with the participation of viral factors at all stages of infection. The synthesis of viral gene products is tightly regulated (Fig. 4): Viral gene expression is coordinately regulated and sequentially ordered in a cascade fashion, with the approximately 70+ gene products forming at least five groups on the basis of both transcriptional and posttranscriptional regulation.

Several of the gene products are enzymes and DNA-binding proteins involved in viral DNA replication. The bulk of viral DNA is synthesized by a rolling circle mechanism, yielding concatemers that are cleaved into monomers and packaged into capsids.

Assembly occurs in stages: After packaging of DNA into preassembled capsids, the virus matures and acquires infectivity by budding through the inner lamellae of the nuclear membrane (Fig. 5) In fully permissive tissue culture cells, the process takes approximately 18–20 hr.

Initial Stages of Infection

The available information on events preceding the transcription of viral genes is still fragmentary. The

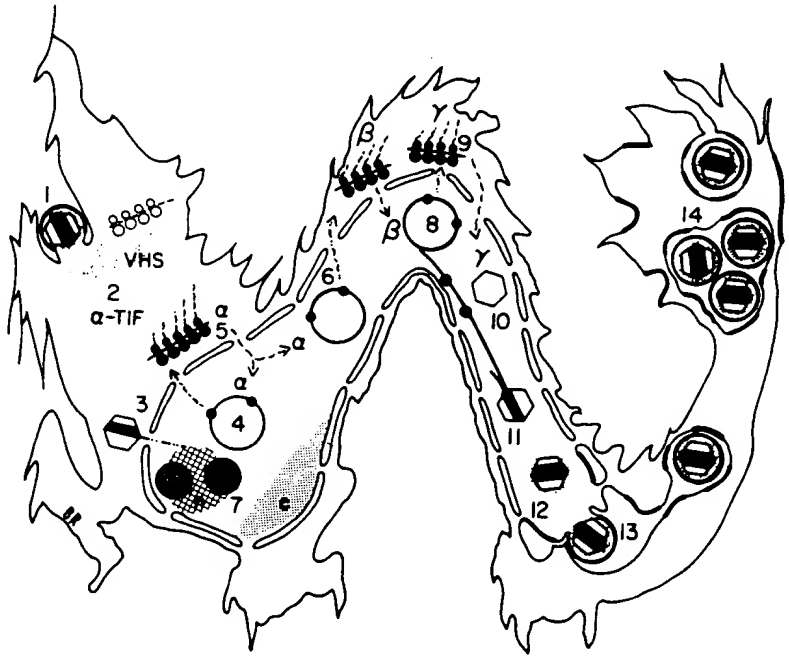

FIG 2. Schematic representation of the replication of herpes simplex viruses in susceptible cells. *1*: The virus initiates infection by the fusion of the viral envelope with the plasma membrane following attachment to the cell surface. *2*: Fusion of the membranes releases two proteins from the virion. VHS shuts off protein synthesis (*broken RNA in open polyribosomes*). αTIF (the α gene *trans*-inducing factor) is transported to the nucleus. *3*: The capsid is transported to the nuclear pore, where viral DNA is released into the nucleus and immediately circularizes. *4*: The transcription of α genes by cellular enzymes is induced by αTIF. *5*: The five α mRNAs are transported into the cytoplasm and translated (*filled polyribosome*); the proteins are transported into the nucleus. *6*: A new round of transcription results in the synthesis of β proteins. *7*: At this stage in the infection, the chromatin (c) is degraded and displaced toward the nuclear membrane, whereas the nucleoli (*round, hatched structures*) become disaggregated. *8*: Viral DNA is replicated by a rolling circle mechanism that yields head-to-tail concatemers of unit-length viral DNA. *9*: A new round of transcription/translation yields the γ proteins consisting primarily of structural proteins of the virus. *10*: The capsid proteins form empty capsids. *11*: Unit-length viral DNA is cleaved from concatemers and packaged into the preformed capsids. *12*: Capsids containing viral DNA acquire a new protein. *13*: Viral glycoproteins and tegument proteins accumulate and form patches in cellular membranes. The capsids containing DNA and the additional protein attach to the underside of the membrane patches containing viral proteins and are enveloped. *14*: The enveloped capsids accumulate in the endoplasmic reticulum and are transported into the extracellular space.

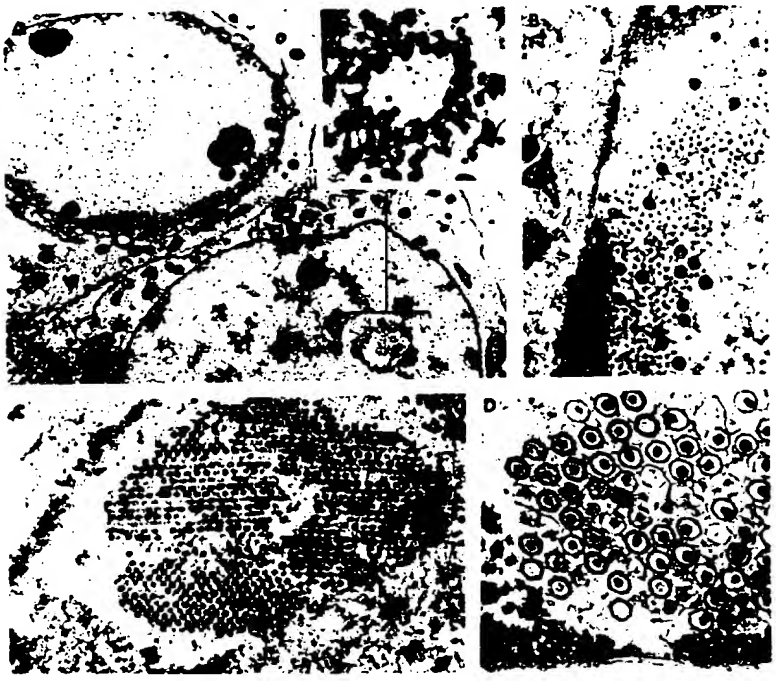

FIG. 3. Electron micrographs of the intracellular events in HSV-1 replication. **A:** Electron-opaque bodies (magnified in insert) showing sites of assembly of capsids. **B:** A region near the edge of the nucleus showing accumulation of chromatin, small particles that appear to be capsid precursors, and capsids. **C:** A paracrystalline array of capsids, both empty and containing DNA, frequently found in nuclei of infected cells. **D:** Capsids in nuclei of infected cells in various stages of packaging of viral DNA. [Electron micrographs have been assembled from refs. 410 and 417 and from J. Schwartz and B. Roizman (*unpublished micrographs*).]

central issue is that two of the initial events—(i) attachment to the cell surface and (ii) fusion of the viral envelope with the plasma membrane—must necessarily involve viral surface proteins. Of the eight HSV glycoproteins, five (gC, gE, gG, gI, and gJ) are dispensable in cell culture, both for entry into cells and for egress from cells (170,273–275,526). As a matter of principle, we do not accept the notion that the virus conserves unnecessary genes or has evolved gratuitous targets for the host immune system. The hypothesis that we raise but do not test is that the five dispensable glycoproteins perform the functions necessary for one or more alternative steps in the initial stages in infection. If that is true, the three essential glycoproteins (gB, gD, and gH) represent the minimal set of surface proteins necessary to sustain and carry out the dominant flow of events. The preferred scenario described in the following sections probably reflects the functions only of this minimal set of surface proteins.

Attachment

HSV-1 and HSV-2 are readily detected on the surface of cells juxtaposed to plasma membranes of cells exposed for a brief interval to infectious virus (Fig. 6). Recent studies by Spear and colleagues (547) indicate that the receptor molecules recognized in one of the initial binding events are heparan sulfate proteoglycans. Consistent with this view, attempts to find cultured cells lacking receptors have not been successful, leaving the species specificity of natural infection by this virus a mystery: other than humans, only chimpanzees are "naturally" infected with this virus (292). Spear and colleagues have demonstrated, by inhibition of attachment of virus by glycoproteins and synthetic peptides, that either gB or gC is required for this step in the process of attachment of HSV to cell surfaces (P. G. Spear, *personal communication*). Whether both glycoproteins see the same domain of heparan sulfate proteoglycans is not yet clear. The attachment of virus to heparan sulfate is the first step in the attachment process.

An apparently similar step in the attachment process is the one that is blocked by the polycations neomycin and polylysine (257,258; G. Campadelli-Fiume, *unpublished data*). Mapping data based on the differences in susceptibility of HSV-1 and HSV-2 to these compounds suggests that this step appears to involve gC. Minson and co-workers (38,99) have recently shown that gH is also required for one of the early steps in viral infection, but this step may involve penetration rather than attachment.

The approach taken in the attachment studies described above is in stark contrast to the many attempts to define the components of the attachment process by analyses of attachment and penetration of viruses exposed to polyclonal or monoclonal antibodies directed against individual proteins. We have set aside the observations that mono- or polyclonal antibodies to each of the major glycoproteins (gB, gC, gD, gE)

may inhibit infection or preclude penetration but not attachment. These catalogues of interesting data may become useful when the precise epitopes and the structures of the surface domains of the glycoproteins become known. In their present form, the data are amenable to many different interpretations.

Penetration into the Infected Cell

Attachment to the cell surface activates a process mediated by viral surface proteins that cause the fusion of the viral envelope and the cell plasma membrane. There is overwhelming acceptance of Morgan et al.'s (322) hypothesis that multiplication results from the entry of virus mediated by fusion of the envelope and plasma membranes rather than from that mediated by phagocytosis. This hypothesis is supported by the observation that penetration by endocytosis results in a nonproductive infection (43). The demonstration that virion envelope Fc receptors [i.e., gE and gI (205)] could be detected on cell surfaces following penetration in the absence of viral gene expression is consistent with this hypothesis (345).

Penetration may be a multistep event involving more than one viral glycoprotein. The cumulative evidence indicates the following: (i) An HSV-1 *ts* mutant expressing an altered gB attaches to, but does not penetrate into, cells (282); however, infection does ensue, and progeny virus is made after chemically induced fusion of the envelope of the adsorbed virus to the plasma membrane (441,442). Consistent with these results, gB^- virus attaches but does not penetrate (41). (ii) HSV-1 gD^- virus also attaches but does not penetrate (206). (iii) Cells expressing HSV-1 gD allow attachment and endocytosis of both HSV-1 and HSV-2; however, fusion of viral and cellular membranes, and

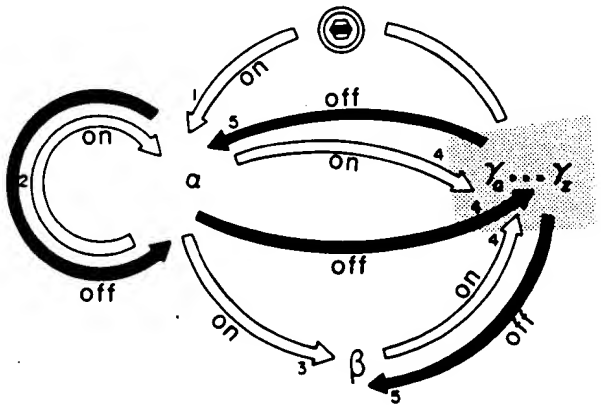

FIG. 4. Schematic representation of the regulation of HSV-1 gene expression. Open and filled arrows represent events in the reproductive cycle which turn gene expression "on" and "off," respectively. *1*: Turning on of α gene transcription by αTIF, a γ protein packaged in the virion. *2*: Autoregulation of α gene expression. *3*: Turning on of β gene transcription. *4*: Turning on of γ gene transcription by α and β gene products through *trans*-activation of γ genes, release of γ genes from repression, and replication of viral DNA. Note that γ genes differ with respect to the stringency of the requirement for DNA synthesis. The heterogeneity is shown as a continuum in which inhibitors of viral DNA synthesis are shown to have minimal effect on γ_a gene expression but totally preclude the expression of γ_z genes. *5*: Turn off of α and β gene expression by the products of γ genes late in infection.

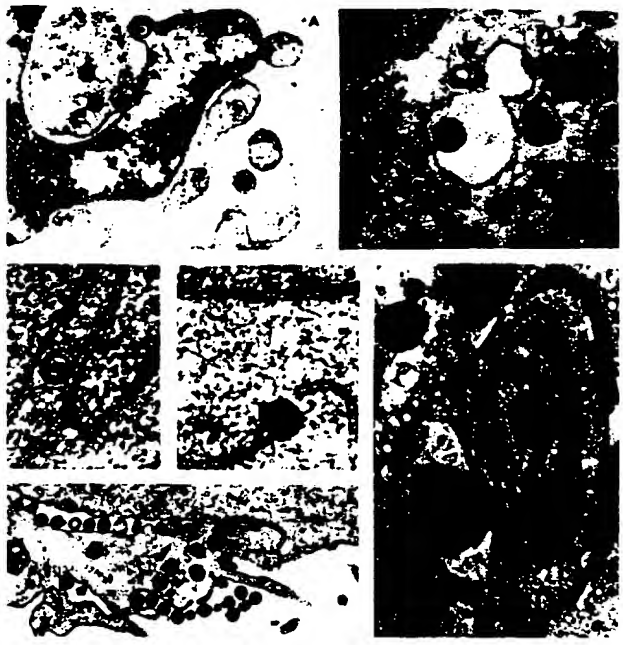

FIG. 5. Electron micrographs of the envelopment and egress of virus from infected cells. **A:** Envelopment of virus from a protrusion of the nucleus. Note that the nucleus contains marginated chromatin. The inner lamellae of the nuclear membrane contain electron-dense, slightly curved patches representing regions of the membrane at which envelopment takes place. Note the spikes projecting from the surface of the membrane of the capsid being enveloped. **B:** An enveloped capsid and numerous unenveloped capsids found late in infection in the cytoplasm of infected cells. Some of the capsids appear to be in the process of being either enveloped or de-enveloped. **C:** Micrograph showing an enveloped capsid in the space between the inner and outer lamellae of the nuclear membrane connecting with the cisternae of the endoplasmic reticulum. **D:** An unenveloped capsid in the nucleus and an enveloped particle bulging in the cisternae of the endoplasmic reticulum. **E:** Cytoplasmic enveloped particles enclosed in vesicles or cisternae of endoplasmic reticulum. **F:** Modified nuclear membranes folded upon themselves frequently seen in cells late in infection. The structures formed by such membranes have been designated as "reduplicated membranes." [Electron micrographs have been assembled from refs. 410 and 417 and from J. Schwartz and B. Roizman (*unpublished micrographs*).]

FIG. 6. Attachment and penetration of HSV-1 to cells in culture. **A** and **B:** Virions attached to plasma membrane. **C:** Capsids with DNA at nuclear pores in cells infected with HSV-1(HFEM)*ts*B7 maintained at the nonpermissive temperature (20). D: Empty capsids accumulating in cells late in infection with mutant HSV-1(50B) late in infection (505). In cells infected with this mutant, virtually every pore contains a juxtaposed empty capsid.

penetration, do not ensue (43). The interpretation of these results is as follows: (a) Both gB and gC recognize, as well as attach to, cell receptors; (b) gB and gD play an indispensable role in the fusion of the envelope with the plasma membrane; and (c) gD sequesters the cell membrane proteins required for fusion of the viral and cellular membranes. Virions attaching to the plasma membrane which cannot fuse are internalized and degraded in endocytic vesicles.

The transition from attached to penetrated virus, as measured by susceptibility to neutralization (characteristic of virus still attached to the cell surface), is very rapid (188).

Release of Viral DNA

Upon entry into the cell, the capsids are transported to the nuclear pores (Fig. 6) (20,505). Release of viral DNA into the nucleoplasm requires a viral function; thus, capsids of the *ts* mutant HSV-1(HFEM)*ts*B7 accumulate at nuclear pores and release viral DNA only after a shift down from nonpermissive to permissive temperature (20). Empty capsids are readily found at nuclear pores early in infection with wild-type viruses. The cellular cytoskeleton probably mediates the transport of herpesvirus capsids to the nuclear pores (80,244). Parental viral DNA accumulates in the nucleus.

Virion Components Required for Replication in Permissive Cells

Transfection of "permissive" cells with intact, deproteinated viral DNA yields infectious viral progeny (160,256,461). However, the specific activity of viral DNA is many orders of magnitude lower than that of virions, and the duration of the reproductive cycle is longer. Moreover, there is no certainty that the sequence of events in transfected cells resembles the viral reproductive cycle occurring in cells infected with competent virions.

The components of the virion other than its DNA appear to have several functions. In addition to protecting and facilitating the entry of the DNA into cells, the virion components appear to be involved in the early shut-off of host macromolecular synthesis (125,252,253,333–335,395,414,443,491,494–496). That virion components also participate in viral replication is deduced from the conclusion that a virion tegument protein (designated in the Spear and Roizman (478) nomenclature as VP16) acts in *trans* to induce α genes, the first set of genes to be expressed (21,46,354,367). Since the induction of α genes is a nuclear event, it is evident that at least some virion components make their way into the nucleus. Preston and Notarianni (376) reported ADP ribosylation of the capsid protein VP23 (148,150,478) in nuclei of freshly infected cells, suggesting that it is also translocated into the nucleus. It has previously been reported that phosphate cycles on and off VP23, suggesting either that VP23 is a component of the protein kinase associated with HSV vi-

rions or that the kinase phosphorylates and dephosphorylates VP23 and substrate proteins (265).

Viral Genes: Pattern of Expression and Characterization of Their Products

Timing and Requirements for Gene Product Synthesis

The transcription of viral DNA takes place in the nucleus. As would be expected, all viral proteins are synthesized in the cytoplasm. The number of abundant (i.e. readily detectable) polypeptides specified by HSV does not exceed 50 (76,183,324). Assuming that only the open-reading frames identified by McGeoch et al. (294,299) are expressed, the HSV genome would encode 70 polypeptides. However, as discussed later in this text, the definition of open-reading frames in the viral genome is somewhat arbitrary, and a higher number is not unlikely.

In cells productively infected with HSV, the regulation of viral gene expression schematically represented in Fig. 4 has three features: (i) HSV proteins form several groups whose synthesis is coordinately regulated in that they have similar requirements for, as well as similar kinetics of, synthesis; (ii) the absolute rate of synthesis and ultimate abundance of each protein may vary; and (iii) the protein groups are sequentially ordered in a cascade fashion (124,183–185, 241,357).

α genes are the first to be expressed. There are five α proteins, namely, infected cell polypeptides (ICPs) 0, 4, 22, 27, and 47. α genes were initially defined as those that are expressed in the absence of viral protein synthesis. The α genes may be defined more precisely by the presence of the sequence 5′ NC GyATGnTAATGArATTCyTTGnGGG 3′ in one to several copies within 400 base pairs upstream of the cap site (280). We should also note that in addition to the five α genes, other domains of the viral genome are transcribed under "α" conditions. The two reported to date are: (i) the latency-associated transcript 1 (LAT1), which is antisense to, as well as partially overlapping, the 3′ domain of the α0 gene (486); and (ii) a transcript designated as ORI_sRNA_1, located in the inverted repeats of the small component with a start site within the 5′ transcribed noncoding domains of the α22/α47 genes, which extends antisense to those genes and terminates approximately at the cap site of the α22/α47 mRNAs (190,191).

The synthesis of α polypeptides reaches peak rates at approximately 2–4 hr post-infection, but α proteins continue to accumulate until late in infection at nonuniform rates (184). To date, all α proteins, with the possible exception of α47, have been shown to have regulatory functions. As discussed in detail below, functional α proteins are required for the synthesis of subsequent polypeptide groups.

β genes are not expressed in the absence of competent α proteins; moreover, their expression is enhanced, rather than reduced, in the presence of inhibitory concentrations of drugs that block viral DNA synthesis or in cells infected with tight DNA^- *ts* mutants in β genes. The β_1 and β_2 groups of polypeptides reach peak rates of synthesis at about 5–7 hr post-infection (184). β_1 genes, exemplified by polypeptides $\beta_1 6$ [the large component of the viral ribonucleotide reductase (192)] and $\beta_1 8$ [the major DNA-binding protein (68)], appear very early after infection and have previously been mistaken for α proteins (61). They are differentiated from the latter by their requirement for functional α4 protein for their synthesis (184,185). β_2 polypeptides include the viral thymidine kinase (TK) and DNA polymerase. The appearance of β gene products signals the onset of viral DNA synthesis, and most viral genes involved in viral nucleic acid metabolism appear to be in the β group.

γ genes have been lumped for convenience into two groups, γ_1 and γ_2, although in reality they form a continuum differing in their timing and dependence on viral DNA synthesis for expression (68,72,176,177, 217,466,515). The prototype γ_1 gene (e.g., the genes specifying glycoproteins B and D) is expressed relatively early in infection and is only minimally affected by inhibitors of DNA synthesis. The relatively abundant major capsid protein, $\gamma_1 5$, is made both early and late in infection. In contrast, prototypic γ_2 genes are expressed late in infection and are not expressed in the presence of effective concentrations of inhibitors of viral DNA synthesis.

γ_1 genes have also been designated as βγ genes (72,176,177). The differentiation of β genes into β_1 and β_2 and the variability in the requirements for the expression of γ genes are the major reasons for the designation of HSV genes as α, β, and γ rather than immediate-early, early, and late (184).

The Functional Organization of Herpes Simplex Virus Genomes

The sources of the data for the functional organization of the HSV-1 genome shown in Fig. 7 are useful to present for both historical and heuristic reasons. Globally, the key sources were the transcriptional maps painstakingly collected and defined by Wagner and associates (5–7,71,72,74,101,102,139,140,162,176, 177,515). These maps served as the basis for the interpretation of the nucleotide sequence data generated by McGeoch and associates (294–300); however, in some instances, transcriptional analyses (and even translational analyses) were ignored in favor of nu-

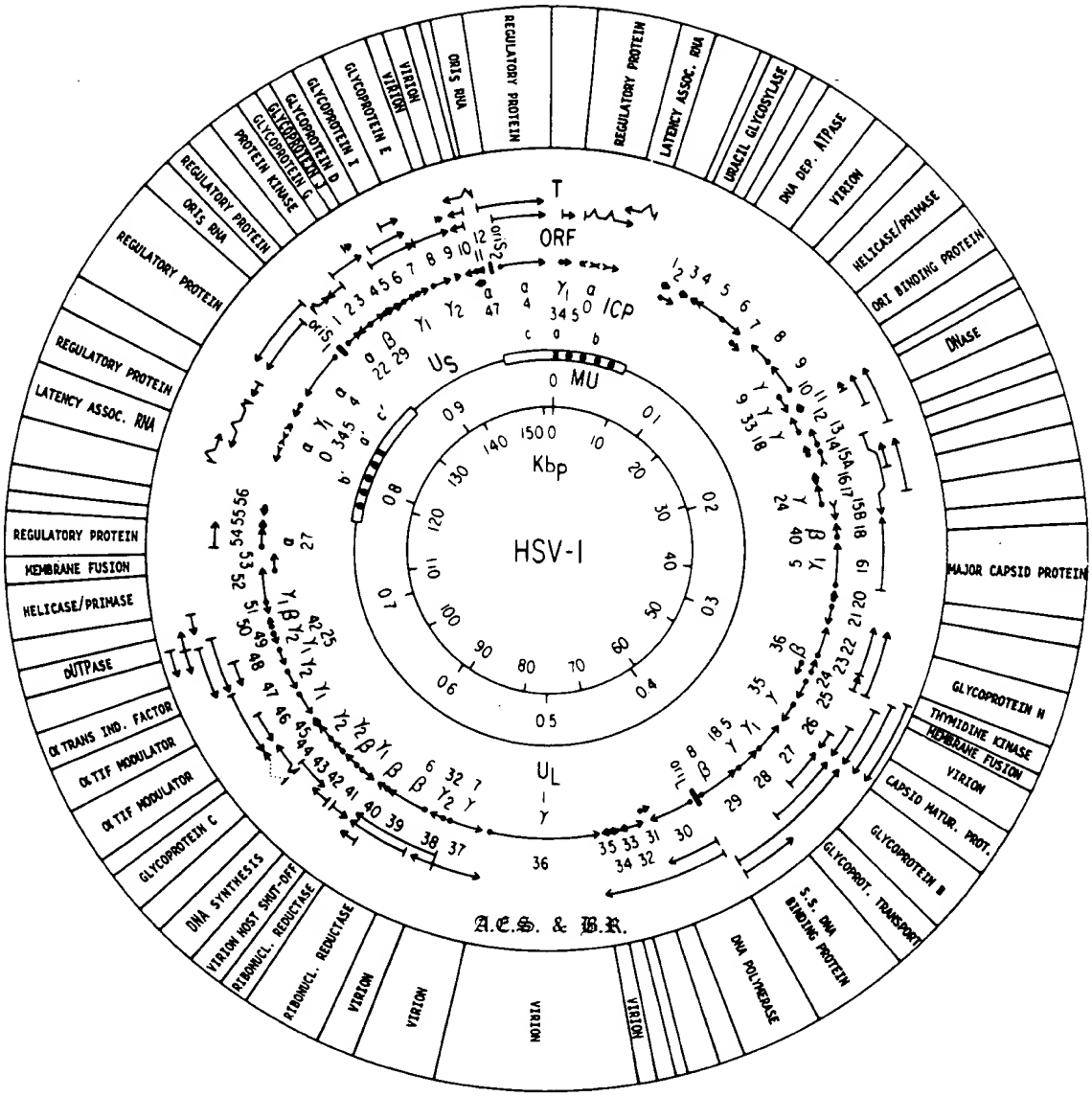

FIG. 7. Functional organization of the HSV-1 genome. The circles are described from inside out. *Circle 1*: Map units and kilobase pairs. *Circle 2*: Sequence arrangement of HSV genome shown as a circularized version of the P arrangement. Cleavage of the circle at O map units would yield a linear molecule in the P arrangement. The letters *a*, *b*, *c*, U_L, and U_s identify different domains of the genome. *Circle 3*: Representation of the open-reading frames. The letters and numbers indicate the regulatory class (α, β, γ_1, or γ_2) to which the gene belongs, and they also indicate the ICP designation of the product. The numbers outside the circle indicate the open-reading frames according to McGeoch et al. (294,298). *Circle 4*: This represents the direction and approximate size of the transcripts as described by numerous laboratories. *Circle 5*: This lists the known functions of the proteins specified by the open-reading frames. Note that virion structural proteins are listed either as "glycoprotein" or "virion." The genes dispensable for growth in cells in culture are listed in the text. The data for circles 3 and 4 are derived from refs. 6, 39, 57, 71, 72, 74, 82, 86, 89, 101, 113, 139, 146, 162, 178, 296–300, 304, 305, 310, 311, 330, 353, 354, 358, 393, 398, 400, 401, 459, 524, and 532. The references for gene functions (circle 5) are listed in the text.

cleotide sequences denoting putative transcriptional initiation sites or terminations. Identification of the proteins specified by the individual open-reading frames is based on several sources. The framework and much of the initial mapping of the HSV genome are based on analyses of proteins and DNA sequence arrangements of HSV-1 × HSV-2 recombinants (286,323,324,434) supplemented by (a) rescue of mutants by transfection of cells with intact mutant viral DNA and DNA fragments generated by restriction endonuclease digestion of wild-type genomes (see, e.g. refs. 232, 233, 326, and 348), (b) transfer of a dominant or assayable marker from one genome to another with restriction endonuclease fragments (see e.g., refs. 232, 240, 367, 368, and 434), and (c) expression of the gene product from purified mRNA or from a DNA fragment in a suitable system (see, e.g., 68, 172, 262, 277, and 367). The products of a large number of putative open-reading frames have not been identified. The sequence-dependent, in contrast to transcription- or function-dependent, identification of open-reading frames is conservative and does not take into account proven exceptions (e.g., the arbitrary rules would have excluded α0 as an open-reading frame if its product had not been known). Nevertheless, the overall organization of the genome is becoming apparent and can be summarized as follows:

1. α genes map near the termini of the L and S components (5,218,277,324,330,378,522,523); α0 and α4 map within the inverted repeats of the L and S components, respectively, and are therefore each present in two copies per wild-type genome. However, a single copy of each is sufficient inasmuch as HSV-1 (I358), a mutant lacking most of the internal inverted repeat sequences, is viable (363). In the circular arrangement of viral DNA, the α genes form two clusters. The first consists of α genes, 0, 4, and 22, whereas the second consists of α genes 47, 4, and 0. A key feature of these two clusters is that each contains an origin of DNA synthesis (Ori_S) sandwiched between α4 and α22 or between α4 and α47. Notwithstanding the clustering, each α gene has its own promoter-regulatory region and transcription initiation and termination sites (277–279).

2. β and γ genes are scattered in both the L and S components. At present, only two functional gene clusters are strikingly apparent, but their significance is uncertain: The β genes specifying the DNA polymerase and the DNA-binding protein flank the L component origin of DNA synthesis (Ori_L), and the γ genes specifying membrane glycoproteins D, E, G, I, and J map next to each other within the unique sequences of the S component (3,130,261,397,419,434,474,475, 524; N. Frenkel, *personal communication*). Although there are several instances of apparent sharing of 5′ or 3′ gene domains (102,398,522), there is altogether little gene overlap and few instances of gene splicing (515) relative to the frequency with which these events have been observed to occur in adenovirus and papovavirus genomes.

In this chapter, each protein shall be designated by one of three criteria: (i) by function, if it is precisely defined (e.g., thymidine kinase, DNA polymerase, etc.), (ii) by the first published designation of the protein, or (iii) by its open-reading frame.

Synthesis and Processing of Viral Proteins

Viral proteins appear to be made on both free and bound polyribosomes. Most of the proteins examined to date appear to be processed extensively after synthesis (2,33,45,112,148,186,357,473–476). Processing includes cleavage, phosphorylation, sulfation, glycosylation, and poly(ADP) ribosylation. In some instances the modifications in protein structure accompany the translocation of proteins across membranes (287). Current information concerning processing of proteins and the relationship of processing to function is detailed in the section on general properties and functions of viral proteins and in the section on viral glycoproteins.

With the exception of some glycoproteins, the extent to which processing is a requirement of virus growth rather than the consequence of a confrontation between cellular or viral enzymes and molecules resembling natural substrates remains uncertain.

It is noteworthy that HSV specifies a protein kinase whose gene maps in the S component (131,297, 388,389). The gene is dispensable for growth in cell culture, and its function is not known. It has been suggested that HSV may also encode a glycosyl transferase, but to date this has not been demonstrated.

Application of Genetic Techniques to the Identification of Gene Product Function: Genes Essential and Nonessential for Growth in Cell Cultures

Key to the identification of viral functions and mapping of viral genes encoding these functions are temperature-sensitive (*ts*) mutants. Some 30 complementation groups have been identified to date (see ref. 529)—an extraordinary accomplishment in itself, given the difficulties inherent in the selection and testing, as well as in the placement of the mutants into complementation groups. The *ts* mutants have been enormously helpful in mapping genes. Nevertheless, this approach to identification and mapping of viral functions suffers from several problems: (i) The phe-

notypes of viruses containing extensive mutations in some nonessential genes cannot be readily differentiated from that of wild-type virus; (ii) conditional lethal (e.g., *ts*) mutants produced by general mutagenesis of the viral genome may contain a large number of silent nonlethal mutations in both essential and nonessential genes; (iii) the phenotypes of mutations introduced into domains shared by more than one gene cannot be readily attributed to the malfunction of a specific gene product; and (iv) while the usefulness of *ts* mutants is, in part, dependent on their efficiency of plating at permissive and nonpermissive temperatures, tight mutants with high permissive/nonpermissive ratios may well contain more than one point mutation. Although the presence of multiple mutations in a single gene should not affect the mapping or identification of the gene function, it does present a problem in mapping the functional domains of the gene.

An alternative to the random or fragment specific substitution of bases in DNA is site-specific deletion of the viral genome. A protocol for site-specific insertion/deletion of viral genes was first reported by Post and Roizman (368). It was based on selection of recombinants generated by double recombination through homologous flanking sequences between an intact viral DNA molecule and a DNA fragment containing an insertion or deletion and a selectable marker. The selectable marker used in these studies was the viral thymidine kinase (*tk*) gene because (i) it can be deleted from the HSV genome without affecting growth of the virus in cell culture, (ii) a plasmid-borne *tk* gene can be altered so that it cannot recombine by double crossover to repair the deletion in the genomic *tk* gene, (iii) viruses carrying a functional *tk* gene can be selected against by plating viral progeny in the presence of nucleoside analogues phosphorylated by the viral TK (e.g., Ara T), and (iv) viruses expressing the *tk* gene can be selected for by plating the virus in TK^- cells in medium containing methotrexate or aminopterin, which block the conversion of TMP from dUMP by thymidylate synthetase and preclude the *de novo* pathway of TMP synthesis. This procedure permits the selection of viable mutants with deletions or insertions in genes that appear to be nonessential for growth in cells in culture. Other investigators adapted the double-crossover protocol for selection of mutants with deletions in essential genes (41,94,267). In this protocol the gene to be deleted was transfected into, and expressed in, Vero cells; the vector cell line (i.e., the cells expressing the gene) was then transfected with intact viral DNA and the mutated DNA fragment. The progeny of transfection were screened for deletion mutants that multiplied only in the vector cell line.

A still different protocol for insertional mutagenesis is based on the use of transposons (e.g., miniMu phage, *Tn5*) (202,418,526). Its principles were described first by Jenkins et al. (202), taking advantage of the random insertion of miniMu into target plasmid DNAs. A miniMu phage containing a modified HSV-1 *tk* gene was constructed. Transposition of this miniMu into an HSV fragment is random and is limited to one insert per plasmid copy. Transfection of intact TK^- viral DNA with an HSV DNA fragment containing random insertions of the modified miniMu would result in recombinants in which the miniMu randomly inserted into the viral DNA fragment would become recombined at the identical position in the viral genome. However, only the genomes containing the miniMu at a nonessential site multiplied in cells in culture.

Among the genome domains not essential for growth in cells in culture are the following: all of the genes mapping in U_s except for that specifying glycoprotein D (273–275,526); the internal inverted repeats (203,274,363); one origin of DNA synthesis in the S component (274); the origin of DNA synthesis in the L component (530); α0 (437,489); dUTPase (128); uracil-DNA glycosylase (327); glycoprotein C (170, 175); the major component of the ribonucleotide reductase (ICP6) (42,154); the minor component of the ribonucleotide reductase (42); the thymidine kinase (223,368); a gene reported to cause fusion of cells (UL24) (196); a gene 3′ to αTIF reported to modulate its activity (UL44) (J. McKnight and B. Roizman, *unpublished data*); and the genes mapping between the 3′ end of α27 and the internal inverted repeats (313). Among the essential genes deleted from the viral genome are those specifying glycoprotein B (41), glycoprotein D (267), α4 (94, 471), α27 (291), and ICP8 (343). As expected, gB^-, gD^-, $ICP8^-$, $\alpha27^-$, and $\alpha4^-$ viruses are not capable of yielding infectious progeny in cells that do not express gB, gD, ICP8, ICP27, or ICP4.

In a special category are deletion mutants whose ability to multiply is cell-species-dependent. One example of such mutants is the $\alpha22^-$ virus, which grows well in Vero and HEp-2 cell lines but not in human fibroblast strains or in rodent cell lines (450). In the nonpermissive cells, the virus fails to express γ_2 genes efficiently.

It could be predicted that viral genes that specify products whose functions are identical and interchangeable with those of cellular genes would be dispensable, at least in cells that express these functions. In this category are the *tk* gene, the genes specifying ribonucleotide reductase, and possibly the gene specifying the protein kinase. It is conceivable that the functions of other viral genes (e.g., those of α22) are complemented by some cells but not by others, and it is also conceivable that some of the dispensable genes may also be complemented by cellular counterparts. However, the dispensable genes specifying the surface glycoproteins C, E, G, I, and J must be in a different

category. It is difficult to ascribe to these proteins functions other than those associated with entry into cells or with egress from cells, functions that are also ascribed to glycoproteins B, D, and H. While we cannot exclude the possibility that cells express proteins with similar functions which complement the deletion mutants, a more likely scenario is that HSV itself carries a set of genes which enables the virus to multiply in a wide variety of human cells. Among these cells may be not only those that do not express *tk*, ribonucleotide reductase, and so on, but also those which require alternate pathways for infection, for regulation of gene expression, and so on. These "nonhomologous" functional alternates remain to be established, but it is relevant to reiterate that both glycoproteins B and C endow the virus with the capability of attaching to heparan sulfate proteoglycans (547).

Synthesis of Viral DNA

Temporal Pattern of Synthesis

A characteristic of herpesviruses not shared by other nuclear DNA viruses is that they specify a large number of enzymes involved in DNA synthesis. Although the sequence of events in viral DNA replication is roughly known, details are lacking. In HSV-infected cells, viral DNA synthesis is detected at about 3 hr post-infection and continues for at least another 9–12 hr (413,414,421). The DNA is made in the nucleus, but the kinetics of DNA synthesis have not been addressed properly. Earlier studies relied on incorporation of labeled thymidine into viral DNA—a procedure that yielded biased results inasmuch as the deoxynucleotide triphosphate pool increases and becomes saturated early in infection. Hence, the rate of viral DNA synthesis as determined by the use of labeled deoxynucleosides appears to be highest relatively early in infection.

Structure of Replicating DNA

At least in HSV-1-infected cells, only a small portion of total input (parental) viral DNA is replicated (194). The DNA labeled during a pulse lacks free ends; that is, it consists of circles or head-to-tail concatemers (194,195). Labeled precursors become incorporated into molecules banding at a higher density which sediment at a faster rate than intact double-strand DNA. In alkaline sucrose density gradients, the bulk of the labeled DNA bands at a position expected for small single-strand fragments. Early after the onset of viral DNA synthesis, parental DNA, circles, and linear branched forms can be found in the DNA banding at the density of viral DNA. These are replaced late in the reproductive cycle by large, rapidly sedimenting bodies of tangled DNA. Available evidence suggests that, at least late in infection, herpesvirus DNAs replicate by a rolling circle mechanism (24,195). Attempts to find "theta" forms of replicating DNA early in infection have not been successful.

Origins of DNA Replication

The origins (*ori*) of DNA replication in the HSV genome (271) were initially deduced from the structures of defective genomes (134,446,471a) and have more recently been operationally defined as those sequences which must be present in a fragment of HSV DNA in order for it to be amplified in permissive cells transfected with the fragment and either transfected or infected with helper virus (319,512). By this definition, HSV-1 and presumably HSV-2 each contain three origins of DNA replication. Two of the origins map in the *c* reiterated sequence of the S component, between the promoters of α4 and α22 (ori_s1) or α4 and α47 (ori_s2) (17,88,319,487,488,512), whereas a third origin (ori_L) maps in the middle of the L component sandwiched between the promoters of the β genes specifying the major DNA-binding protein (ICP8) and the DNA polymerase (271,472,530).

The L-component origin consists of an A + T-rich, 144-base-pair sequence forming a perfect palindrome (234,272,393,530). Because of its extensive dyad symmetry, it tends to be unstable in DNA fragments cloned in *Escherichia coli* (530). The S component origin is shorter and contains a much shorter A + T-rich palindrome which is related to, but lacks, the complete dyad symmetry of ori_L. It has been suggested that the structure of ori_L enables bidirectional synthesis, whereas DNA synthesis initiated in ori_s would be asymmetric. The existence or necessity for bidirectional synthesis of DNA remains to be established. Ori_L and at least one ori_s can be deleted without affecting the ability of the virus to multiply.

Very little is known about the function of the origins. The conclusion that these sequences are indeed origins is supported by the studies of origin-dependent amplification of DNA by fragments encoding genes shown to be essential for viral DNA synthesis. However, major questions remain unanswered, particularly the function of the origins once viral DNA synthesis is initiated and whether the two kinds of origins are equivalent or subordinate to each other, especially because the reported positions of loops in replicating HSV DNA appear, in some instances, to be different from those of the known origins (465). It is noteworthy that both ori_L and ori_s are situated between transcription initiation sites. The locations of the origins suggest that initiation of DNA synthesis might be activated, or

at least enhanced, by the changes in the local environment of the DNA due to transcription initiation events.

Functional Requirements for DNA Synthesis

HSV specifies a large array of proteins involved in nucleic acid metabolism and DNA synthesis. These proteins fall into two categories: (i) proteins that are essential for viral origin-dependent amplification of DNA and (ii) enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, ribonucleotide reductase, dUTPase, uracil-DNA glycosylase, alkaline exonuclease) which, for the most part, appear not to be essential for viral growth in cells in culture. A virus-specific topoisomerase has also been reported (329) but has not been shown to be virally encoded (16).

Much of the initial evidence for viral proteins essential for DNA synthesis emerged from studies of the defects in DNA$^-$ *ts* mutants. More recently, the genes whose products are essential for DNA synthesis were identified by transfecting cells with a plasmid containing an origin of DNA synthesis and various fragments of the HSV genome. These studies (53) identified seven genes mapping in the L component (open-reading frames UL5, UL8, UL9, UL29, UL30, UL42, and UL52) required for viral-origin-dependent DNA synthesis. The seven genes specify the following: a DNA polymerase (UL30) with an apparent molecular weight of 140,000 (55,62,167,182,220,221,369); a single-strand specific-DNA-binding protein designated as ICP8 (UL29) with an apparent molecular weight of 124,000 (53,59,68,204,237,283,288,369,390,435,546); a protein binding to the origin of viral DNA synthesis (UL9) (106,107,236,340) with a translated molecular weight of 94,000 (107); a protein that binds to double-strand DNA (UL42) with a molecular weight of 62,000 (285,294,347,546); and three additional proteins (UL5, predicted molecular weight of 99,000; UL8, predicted molecular weight of 80,000; and UL52, predicted molecular weight of 114,000). These three proteins form a complex in which each protein is present in equimolar rations and which functions as a primase and a helicase (78). An HSV-specific primase activity was previously reported by Holmes et al. (179). The protein specified by UL5 has independently been shown to be a DNA-dependent ATPase (E. Mocarski, *personal communication*).

The DNA polymerase, in particular, has been the object of numerous studies because of its unusual sensitivity to a variety of compounds (e.g., phosphonoacetate and phophonoformate). Temperature-sensitive mutants expressing altered HSV DNA polymerases have been described (55,62,386,387), and some mutants have been found to be resistant to a variety of drugs such as phosphonoacetate (54,204,386) and nucleoside analogues [e.g., acycloguanosine (77)] inhibitory to wild-type viruses.

ICP8 ($\beta_1 8$) has also been extensively investigated, particularly by Knipe and colleagues (151–153,237,259,260,390–392), and by Ruyechan and colleagues (431–433,435). The protein has an apparent molecular weight of approximately 120,000. It has an affinity for single-strand DNA (385), and its binding is cooperative (431). Temperature-sensitive mutants in this gene fail to synthesize viral DNA at the nonpermissive temperature (68,151), as do deletion mutants in cells that do not provide ICP8 in *trans* (343).

The seven proteins described above appear to be all that is necessary for ori$_s$-dependent amplification of DNA transfected into cells. Other proteins undoubtedly play a role in processing, cleavage, and packaging of the genomic viral DNA, as well as in the production of precursors of DNA synthesis; for example, as described below, the alkaline DNase is not among these seven genes but is essential for viral DNA replication (325).

Alkaline DNase

HSV-1 induction of an alkaline DNase activity was first reported in 1963 (221). The gene has been mapped by transient expression in oocytes (373) and by the use of HSV-1 × HSV-2 recombinants (14) to between 0.145 and 0.185 map units, corresponding to open-reading frame UL12 (294). The protein is encoded by a 2.3-Kb mRNA (101) and has a predicted translated molecular weight of 67,503 (294) and an apparent molecular weight of 80,000–85,000 (15). A *ts* mutant (132) was used to demonstrate that the DNase activity is essential not only for viral growth but also for DNA replication (325). In contrast to the requirement for this activity for DNA synthesis in infected cells, the enzyme is not required in transient expression assays involving individual viral genes and a viral origin of DNA synthesis contained in a plasmid (53).

Several other proteins involved in nucleic acid metabolism have been described but appear not to be essential for virus growth in cells in culture.

Thymidine Kinase (TK)

TK is by far the best known of the viral proteins. A unique characteristic of TK is that its substrate range is far greater than that of its host counterpart. Although it has been designated as a deoxypyrimidine kinase (198), it actually phosphorylates purine pentosides and a wide diversity of nucleoside analogues that are not phosphorylated efficiently by cellular kinases (204, 224,231). This characteristic of TK is the basis for the

effectiveness of various nucleoside analogues in the treatment of experimental and natural herpesvirus infections. The observation that TK is essential for normal virus multiplication in experimental infections (127,500) but not in cell culture (223) is the basis of much of the probing of the HSV genome structure done in recent years (363,367,368). Mutants in the *tk* gene fall into several groups. Some fail to produce functional TK altogether, whereas others make either (a) reduced amounts of enzyme or (b) an enzyme with an altered substrate specificity which is resistant to the analogue used in the selection process (83,127, 370,492).

Ribonucleotide Reductase

The HSV ribonucleotide reductase consists of two proteins: The large subunit, ICP6 (184,185,192,380), has an apparent molecular weight of 140,000 and a predicted translated molecular weight of 124,043 (294); the small subunit has an apparent molecular weight of 38,000 (11,380) and a predicted translated molecular weight of 38,017 (294). The two genes are encoded by 3'-coterminal mRNAs of 5.0 Kb for the large subunit and 1.2 Kb for the small subunit (6,493). The two proteins are tightly associated in a $\alpha_2\beta_2$ complex (11,12,193), and both subunits are required for activity (11,129,187).

Ribonucleotide reductase functions to reduce ribonucleotides to deoxyribonucleotides, creating a pool of substrates for DNA synthesis. The viral enzyme is not essential for growth in actively dividing cells maintained at 37°C (154). However, it is required for efficient viral growth and DNA replication in nondividing cells or in cells maintained at 39.5°C (155,380), indicating that at 37°C, actively dividing cells can complement the viral function.

Uracil-DNA Glycosylase

HSV encodes a uracil glycosylase, which presumably functions in DNA repair and proofreading. Uracil glycosylase acts to correct the insertion of dUTP and to correct the deamination of cytosine residues in DNA; the extremely high G + C content of HSV DNA makes this an important element of error correction in HSV DNA replication. The HSV-induced uracil glycosylase has been identified by Caradonna and Cheng (47), and its coding domain was initially mapped to between 0.065 and 0.08 map units (48), a location correlating to the UL2 open-reading frame (294). Subsequent *in vitro* translation experiments definitively identified UL2 as the uracil glycosylase gene (545). The protein has an apparent molecular weight of 39,000 (48) and a predicted translated molecular weight of 36,326 (294). The gene has been deleted and is nonessential for growth of the virus in culture (327).

dUTPase (Deoxyuridine Triphosphate Nucleotidohydrolase)

dUTPases act to hydrolyze dUTP to dUMP, providing both (a) a mechanism to prevent incorporation of dUTP into DNA and (b) a pool of dUMP for conversion to dTMP by thymidylate synthetase. An HSV-encoded dUTPase has been identified (47,543); contrary to early reports (543), the purified enzyme is specific for the hydrolysis of dUTP (541). The viral gene has been mapped to between 0.69 and 0.70 map units by transient expression (379), corresponding to the UL50 open-reading frame (294). dUTPase activity appears to be lacking in HSV-1(17)*ts*K, a mutant in ICP4 used for analyses of gene regulation (84). The dUTPase gene has subsequently been shown to be nonessential for growth of the virus in tissue culture (128).

Assembly of Capsids

Capsids are assembled in the nucleus (Fig. 3). The steps in the assembly are not defined. Viral DNA is packaged into preformed capsids.

Cleavage and Packaging of HSV DNA

Newly synthesized viral DNA is "processed" and packaged into preformed empty capsids. "Processing" involves (a) amplification of *a* sequences and (b) cleavage of viral DNA lacking free ends (i.e., in circular or head-to-tail concatemeric form). The isomerization of the DNA is associated with the process of DNA replication, cleavage, and packaging. There is considerable evidence that cleavage and packaging of DNA are linked processes (90,91,254,255). The isomerization of the DNA is less well understood. The available data have come from three sources: (i) analyses of the termini of standard viral genomes (87,321,323); (ii) analyses of termini of viral genomes containing insertions of additional *a* sequences (56,510); and (iii) studies on amplicons, plasmids containing an origin of viral DNA synthesis and one or more *a* sequences which are amplified and packaged with the aid of a helper virus (90,91,472,512,513).

The net result of the process of cleavage of standard genomes from concatemers is the generation of (a) a free S-component terminus consisting of one *a* sequence with a terminal DR1 sequence containing only a single base pair and and one 3' nucleotide extension (320) and (b) a free L-component terminus consisting of one to several directly repeated *a* sequences and

ending in a DR1 containing 18 base pairs and one 3′ nucleotide extension. Upon circularization of the DNA following entry into cells, the two partial DR1 sequences together would form one complete DR1 shared by two *a* sequences. In the reverse process of linearization of viral DNA for packaging, cleavage of endless (circular or concatemeric) DNA occurs asymmetrically within a DR1 second distal from the *c* sequence and, in an ideal case, shared by two *a* sequences. Studies on junctions containing a single *a* sequence show that they are cleaved (91). The results of such studies have been interpreted to indicate either (a) that the sequence *xay* is cleaved to yield *xa* and *y*, and the *y* product is processively degraded along the DNA to the next *a* sequence, or (b) that the cleavage simultaneously yields both *xa* and *ay* by amplification of the *a* sequence during the cleavage process (90,91,510). Parenthetically, there is little doubt that DNA lacking a terminal *a* sequence could be degraded, but inasmuch as nearly 50% of the L–S component junctions are of the *bac* type (i.e., have a single *a* sequence), a hypothesis whose logical extension is that 50% of newly synthesized DNA is degraded during packaging does not make biological sense.

Deiss et al. (90) analyzed the cleavage and packaging of a series of amplicons. Those lacking the *Ub* sequence were amplified and packaged, but they acquired an intact *a* sequence from the helper virus. Those lacking the *Uc* sequence were not subject to cleavage and packaging. Domains were identified in *Ub* and *Uc* which were conserved in several herpesviruses and which were designated *pac1* and *pac2*, respectively. The model (90) that best fits the data, presented here in a slightly modified form (Fig. 8), consists of several steps: (i) A cleavage–packaging protein attaches to the *Uc* sequence. (ii) A putative structure on the surface of the capsid complexes with a *Uc*-bound protein sequence, loops the viral DNA, and scans from the bound *a* sequence (a_1) across the L component toward the end of the S component until it detects the first Uc-DRI-Ub domain of an *a* sequence in an identical orientation. (iii) In the juxtaposed *a* sequences, the DR1 sequence of one *a* is cleaved and the gap is repaired, resulting in the generation of an *a* sequence by the mechanism proposed by Szostak et al. (497) to explain recombinational events resulting in gene conversion. (iv) Cleavage then occurs in the DR1 shared by the two *a* sequences. In this model, the *a* sequences in the internal inverted repeats play no role in the packaging of the unit-length molecule, consistent with the observation that HSV-1 DNA from which the internal inverted repeats are deleted do package effectively.

The packaging component of the model of Deiss et al. (90) predicts that the length of the packaged DNA can be defined by the distance between two directly repeated *a* sequences. The studies by Frenkel et al.

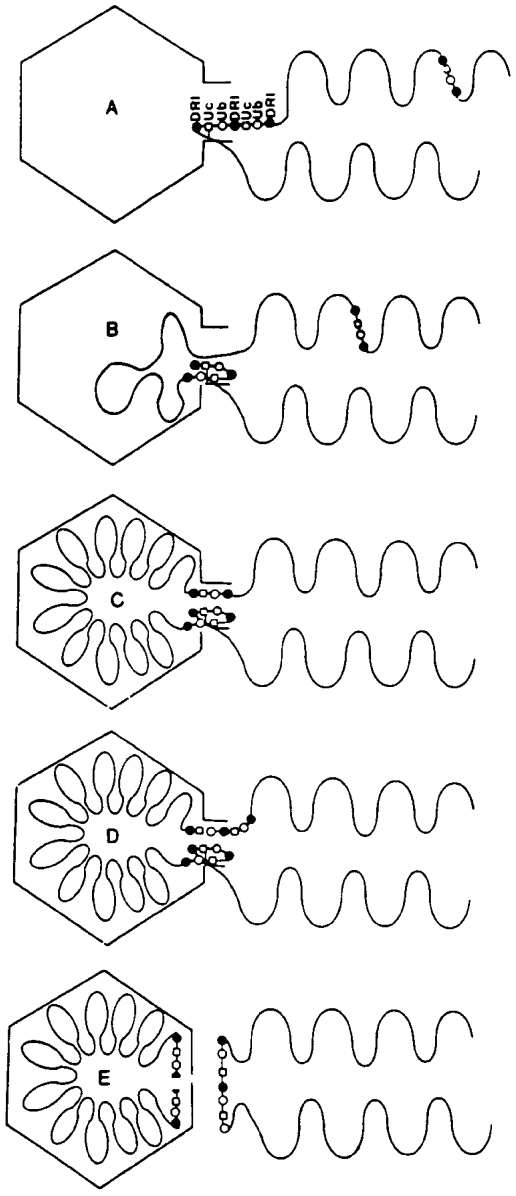

FIG. 8. Packaging of HSV-1 DNA. The current model developed by N. Frenkel and associates is described in Deiss et al. (90) and in the text. The model requires that proteins attach to components of the *a* sequence, probably Uc, and that empty capsids scan concatemeric DNA until contact is made in a specific orientation with the first protein–Uc sequence (**capsid A**); the DNA is then taken into the capsid (**capsid B**) until a "head full" is taken in or one *a* sequence whose nucleotide arrangement is in the same orientation (i.e., one genome equivalent in length away) is encountered (**capsid C**); the packaging signal requires nicking of both strands from signals on opposite sites of a DR1 sequence. In the absence of two adjacent *a* sequences (**capsid D**), the juxtaposition of the *a* sequences would result in duplication of the *a* sequence (**capsid E**) as described by Deiss et al. (90)

(134) indicate that the situation is likely to be more complex. Defective genomes consisting of 17+ direct reiterations of a unit consisting of an *ori* and an *a* sequence are readily detected in virions of HSV stocks derived by serial passages at relatively high multiplicities (134). These observations are consistent with the hypothesis that, besides the scanning mode, there is a "head full" recognition element that selects the juxtaposed *a* sequence once a threshold amount of DNA has been packaged. There is evidence, however, that shorter fragments of HSV DNA are packaged into capsids but that these capsids do not become enveloped (513). The hypothesis that may explain the apparent contradiction is as follows: Packaging aborts when the DNA reeled into the capsid is smaller than full length, but the capsid does not disgorge the packaged fragment.

The viral proteins responsible for the cleavage–packaging event have not been identified, but two sets of DNA-binding proteins of potential significance have been reported. Chou and Roizman (58) have identified two viral proteins which form a sequence-specific complex with the portions of the *Uc* sequence containing *pac2*. In addition, capsids contain a protein that binds viral DNA [VP19C or ICP32 (32)].

Inversions of the L and S Components

The isomerization of HSV DNA resulting from the inversion of the L and S components relative to each other is an intriguing, tantalizing feature of the HSV genomes shared with only a few other herpesviruses.

In its circular form, the HSV genome forms two isomers, each containing two L–S component junctions. Cleavage of one circular isomeric form at the two junctions would yield the P and I_{SL} arrangements, whereas the corresponding cleavages of the other circular isomer would yield the I_S and I_L isomers. Generation of the I_S and I_L arrangements from the first circular isomeric form would require inversion of either the S or the L component through the inverted repeat sequences.

Fundamentally, there are several issues. First, inversion of covalently linked components is not a property of all herpesvirus genomes. Second, the physiologic function of the inversions is not clear inasmuch as genomes frozen in one orientation as a consequence of deletion of internal inverted repeats are viable (203,362). However, all wild-type isolates examined to date do contain the inverted repeat sequences, and viruses lacking internal inverted repeats have a reduced capacity for growth in animal tissues. Third, insertion of the junction between the L and S components, and especially of the 500-base-pair *a* sequence, results in additional inversions of DNA segments contained between inverted repeats of *a* sequences (56,317, 321,323). Deletion analyses have shown that inversions are associated with the sequences DR2 and DR4; deletion of these sequences results in a gross reduction in the inversion frequency (56). Lastly, inversion of viral DNA segments flanked by other domains of the genome, or inversion between repeated foreign DNA sequences, was observed in some instances but not in others. In the case of fragments duplicated in different components of the HSV DNA, the segment of the genome flanked by the inverted repeats does not invert (317,368). DNA fragments flanked by inverted repeats contained in the same component do invert. In some instances, the inversions were accompanied by a high-frequency gene conversion (365). Recently, Weber et al. (525) reported that inversions of DNA segments flanked by inverted Tn5 transposon elements resulted from recombination events through homologous sequences and was not the consequence of a recombinational event mediated at a specific *cis*-acting site by *trans*-acting viral proteins. Thus, inversions of amplified DNA sequences flanked by inverted Tn5 sequences at least 600 base pairs or longer were noted in cells transfected with the genes specifying the seven proteins required for viral DNA synthesis. As in the case of amplicons containing two *a* sequences in an inverted orientation (319), inversions were not observed in the absence of DNA synthesis. Experiments with HSV L–S junctions were also done, but only with direct repeats of the *bac* sequence. In this instance, the intervening segment was deleted.

The central issue is not that DNA sequences flanked by inverted repeats tend to invert as a consequence of homologous recombination, but rather the frequency of such inversions. The DNA extracted from a plaque, generated by a single virus particle presumed to be in one arrangement of DNA, contains all four isomers of HSV DNA in equimolar concentrations. In the case of DNA segments flanked by inverted repeats of nonjunction fragments, the fraction of the genomes showing inversions even after many serial passages is seldom more than a small fraction of the total. The accumulated and predicted evidence that DNA segments flanked by inverted repeats in HSV genomes can invert does not resolve the mystery of the high-frequency inversion of the L and S components relative to each other during viral replication.

Envelopment

Entry of Viral Proteins into Cellular Membranes

The hallmark of infected cells late in infection is the appearance of reduplicated membranes and thick, concave or convex patches, particularly in nuclear mem-

branes (Fig. 5) (69,111,263,293,321,332,447,464). Nuclear envelopment takes place at these patches. Because the enveloped virions do not contain detectable amounts of host membrane proteins, it is likely that the patches represent aggregations of viral membrane proteins, presumably including (a) the viral glycoproteins on the outside surface and (b) anchorage and tegument proteins on the inside surface.

A central, puzzling issue is the mechanism of transport of viral glycoproteins to the nuclear membranes. A no less intriguing question involves the nature of the interaction of viral membrane proteins among themselves: Do they form well-defined structures, or do they float freely in the lipid bilayer?

To date, the viral membrane proteins identified in HSV-infected cells have been mostly glycosylated surface proteins. Analyses of the predicted structures of proteins based on the nucleotide sequence of open-reading frames suggest that the HSV-1 genome may specify other proteins that could traverse the lipid bilayer several times (e.g., the products of open-reading frames UL10, UL20, UL43, and UL53) (294).

Available evidence suggests that the general pattern of the biosynthesis of herpesvirus glycoproteins follows that of eukaryotic cell glycosylated proteins (45,474). Specifically, nonglycosylated precursors of herpesvirus membrane proteins are synthesized on polyribosomes bound to the rough endoplasmic reticulum. Glycosylation includes translational and post-translational events. Thus, *N*-glycosylation is initiated by transfer of preformed glycans [$(glucose)_3$-$(mannose)_9$-(N-acetylglucosamine)$_2$] from a dolichol phosphate lipid carrier to asparagine residues in the sequence Asn-X-Thr/Ser (X can be almost any amino acid) of a nascent polypeptide (238). During transit to the Golgi bodies, the oligosaccharide chains are trimmed by glucosidases and mannosidases to yield a glycan with five mannose and two *N*-acetylglucosamine residues. The high-mannose glycans are frequently converted by glycosyl transferases located in the *trans*-Golgi to complex glycans that consist of a pentasaccharide core [$(mannose)_3$-N-acetylglucosamine$)_2$] and a number of side chains (antennae) with the overall composition of sialic acid–galactose–*N*-acetylglucosamine. Fucose, when present, is usually added to the completed side chains (see ref. 238). O-linked glycosylation occurs less frequently than N-linked glycosylation (45,209,341,474). In some glycoproteins, notably gC of HSV-1 and gG of HSV-2, the O-linked oligosacharides are the major components (81,457); in other glycoproteins (e.g., gD of HSV-1 and HSV-2), however, the N- and O-linked oligosaccharide chains are present in roughly equal numbers (455). As is the case for many other mammalian glycoproteins (see ref. 25), O-linked glycosylation is initiated by the transfer of *N*-acetylgalactosamine to the hydroxyl group of threonine or serine and is followed by the addition of galactose and one or two sialic acids in the Golgi apparatus (81,456).

Current information on the structure of the HSV glycans has been summarized in detail elsewhere (45,474). Thus, all types of glycans (N-linked high-mannose; N-linked complex type; and O-linked) have been reported to exist in HSV-1 glycoproteins. HSV-1 glycoproteins are characterized by microheterogeneity in the glycans they carry, due to differences in the extent of chain processing and sialylation. Microheterogeneity has been shown even at a single *N*-glycosylation site (45,455). The first O-linked sugar, *N*-acetylgalactosamine, is added to the glycoproteins that carry N-linked oligosaccharides poorly processed by mannosidases, prior to their routing to the Golgi (454). Processing of HSV glycoproteins is carried out mainly by the host glycosylation machinery inasmuch as viral glycoproteins made in cells defective in some glycosylation enzymes reflect the defect (44,456). However, the available data do not exclude the possibility that the virus specifies one or more enzymes whose functions are similar to those of the host.

Nothing is known regarding the function and requirements for O-linked glycosylation of herpesvirus glycoproteins. Whereas virus particles containing high-mannose glycans are infectious, at least the initial step in N-linked glycosylation is required for infectivity inasmuch as blocking of N-linked glycosylation by tunicamycin blocks the accumulation of glycosylated proteins and of enveloped virus (352,359,360). Conversion of high-mannose glycans into complex-type glycans appears to be required for the egress of the virus from the infected cell (44,208,239,456). There is considerable evidence that after synthesis the viral glycoproteins are transported to the plasma membrane and can be found in cytoplasmic membranes of the cell. The viral glycoproteins in the cellular membranes are the targets of the immune response to the virus. Viral glycoproteins specified by genes resident in the environment of the cells mature and are transported faster than the proteins specified by genes resident in the viral genome and expressed during infection. The difference may simply reflect timing and intracellular traffic congestion. In cells expressing both a glycoprotein gene resident in the cellular genome and the corresponding gene resident in the viral genome, the former gene is expressed earlier; the glycosylation and transport of this protein does not compete with that of abundant viral glycoproteins made later from transcripts of genes resident in the viral genome (10).

Nothing is known of the mechanism by which viral glycoproteins enter nuclear membranes. As noted below, it has been suggested that virions enter the Golgi apparatus after envelopment. In polarized kid-

ney cells, viral glycoproteins are sorted to the basolateral membrane.

Several lines of evidence suggest that HSV membrane proteins form specific complexes. The existence of one complex, the Fc receptor formed by gI and gE, can be deduced from the observation that monoclonal antibody to either precipitates both glycoproteins (205). Another observation relates to an aberrant property of some mutants of HSV-1 and HSV-2 which cause infected cells to fuse.

Both HSV-1 and HSV-2 cause infected cells to round up and cling to each other. Some viral mutants cause cells to fuse into polykaryocytes; this fusion may be cell-type-specific or cell-type-independent (105,175, 434). Polykaryocytosis has been studied for several reasons: (a) as a probe in the structure and function of cellular membranes, reflected in the "social behavior of cells," (b) as a tool for analyses of the function of viral membrane proteins, and (c) as a model of the initial interaction between HSV and susceptible cells that results in the fusion of the viral envelope and the plasma membrane (45,407,408,474). Cell fusion induced by HSV requires full processing of high-mannose glycans to complex glycans up to the addition of sialic acid. In this instance it is not clear whether sialilated glycans must be present (a) only in viral glycoproteins located on the surface of the infected recruiter cells or (b) in the recruiter cells as well as in the uninfected cells to be recruited in polykaryocytes (45,474).

Polykaryocytosis can be viewed as an aberrant manifestation of the interaction of altered membrane domains of infected cells and unaltered membranes of juxtaposed cells (407,408). Genetic analyses have shown that mutations (*syn*) which confer the capacity to fuse cells map in at least four (and possibly more) loci within the viral genome (31,89,269,364,366,431, 439,548). Only one of these loci is within the domain of a viral glycoprotein (gB) and has been mapped in the carboxyl-terminal, cytoplasmic domain of the protein. One interpretation of this observation is as follows: The membrane proteins form complexes whose structure and conformation become altered by mutations in any of the component polypeptides, and the changes in conformation are similar to those which occur in the envelopes of virions interacting with the plasma membrane (434).

Envelopment

Nuclear DNA-containing capsids attach to patches of modified inner lamellae of the nuclear membrane and become enveloped in the process. The emphasis on "DNA-containing" capsids is a result of electron-microscopic observations which show that envelopment of empty capsids occurs rarely, although there is no evidence that "full" capsids contain a full-length molecule of HSV DNA (417). As discussed above, Vlazny et al. (513) demonstrated that capsids containing fragments of HSV DNA less than standard genome length are retained in the nucleus. A plausible explanation for this phenomenon rests on the observation that DNA-containing capsids differ from empty capsids with respect to protein VP22 (ICP35) (32,34,150). Conceivably, capsids become modified during packaging of the DNA, and only modified capsids are able to bind to the underside of the thickened patches containing viral proteins in the nuclear membranes.

There is general agreement that the inner lamellae are the site of initial envelopment (Fig. 5). However, even cursory examinations of thin sections of infected cells elicits the rediscovery that envelopment occurs in the cytoplasm, since the cytoplasm contains capsids juxtaposed to patches of modified cytoplasmic membranes in the process or envelopment or de-envelopment. Stackpole (479) is the originator of the idea that the capsids become enveloped at the inner lamellae, de-enveloped at the outer lamellae, re-enveloped by the endoplasmic reticulum, and released in the extracellular environment either by envelopment at the plasma membrane or by fusion of vesicles carrying enveloped virus at the plasma membrane. The large number of particles seemingly undergoing cytoplasmic envelopment makes a strong case for this model. However, thin sections showing capsids being enveloped at the nuclear membranes are extremely rare, suggesting that the process of envelopment is very rapid. Since every capsid undergoing putative envelopment in the cytoplasm must have been enveloped and de-enveloped in transit through the nuclear membranes, the disparity in the numbers of capsids being enveloped at the nuclear and cytoplasmic membranes suggest that either (a) the rate of envelopment at the nuclear membranes is significantly faster than that at the cytoplasmic membranes or (b) the capsids in juxtaposition to cytoplasmic membranes are artifacts and represent capsids that are de-enveloped or arrested in their movement through membranes. The key question of whether the cytoplasmic, semienveloped capsids are in fact in the process of being enveloped (rather than being arrested, transient structures resulting from de-envelopment) remains unanswered. One hypothesis for the presence of partially enveloped structures at cytoplasmic membranes is that virions contained in the endoplasmic reticulum attach to receptors and reinfect the cell from within, but the capsid is not transported to the nuclear pore for lack of cytoskeletal connections. It is of interest that cytoplasmic semienveloped capsids are prevalent in continuous cell lines but

are less prevalent in infected primary human diploid cells.

Transit Through Cytoplasm; Egress and Re-entry

In the cytoplasm, intact enveloped particles are usually seen inside structures bounded by membranes (85,448). This observation is not unpredicted, inasmuch as structures bounded by membranes with surface glycoproteins are not likely to fare well unprotected in the cytoplasm. In two-dimensional sections, these structures appear to be vesicles. These vesicles may well represent the vehicles by which virions transit through the cytoplasm. In a few electron micrographs, tubular structures have been seen, and the probability exists that in some cells the cisternae of the endoplasmic reticulum extend to the plasma membrane (448). Any model for cytoplasmic transit of virions that culminates in egress must take into account that (i) all conditions which lead to a block in glycoprotein maturation hinder virus egress and induce an intracytoplasmic accumulation of enveloped particles containing immature glycoproteins (45,239,474), and (ii) nuclear membranes appear to contain predominantly immature forms of glycoproteins (67). It has been suggested that virus egress is achieved by a mechanism akin to reverse phagocytosis, a term which conveys the direction (rather than the mechanics) of the motion. Thus, Johnson and Spear (208) proposed, in part on the basis of studies done with monensin, that virions are secreted via the Golgi apparatus following a pathway similar to that taken by secreted soluble proteins.

In cells infected with a mutant virus containing a lesion mapping in the S component, but not in cells infected with wild-type virus, empty capsids accumulate in large numbers at the outer surface of the nuclear membrane, suggesting the possibility that HSV encodes a function to prevent reinfection of cells, particularly with virus which had been released from these cells (505). This function has been attributed to glycoprotein D (43).

Membrane Proteins

In addition to the glycycoproteins listed earlier in the text, several other membrane proteins have been postulated to exist on the basis of mapping of *syn* mutants described below. Furthermore, analyses of the structure of putative proteins predicted to exist on the basis of the HSV-1 DNA sequence (294) have identified several proteins whose predicted structures are consistent with those of multimembrane spanning proteins. These are the proteins predicted to be encoded in the open-reading frames UL10, UL20, UL43, and UL53. Other proteins with the potential to interact with membranes are UL34, UL45, and US9 (294).

REGULATION OF VIRAL GENE EXPRESSION

Structure of HSV mRNAs

The properties of viral mRNAs are central to a discussion of the regulation of viral gene expression. HSV DNA is transcribed by RNA polymerase II (75). Viral mRNAs are capped, methylated, and polyadenylated, although nonpolyadenylated RNAs of the same sequences can be isolated (13,18,19,467,469,490). Internal methylation is readily apparent in RNA made early, but not late, in infection (19). Notwithstanding the efficient expression of HSV genes in the environment of higher eukaryotic cells, only a relatively small proportion of HSV mRNAs are derived by splicing. Genes sharing 5′ (or, particularly, 3′) termini have been described (310,515). Attention has also been drawn to (a) multiple initiation sites for the transcription of selected HSV genes (139,330,459,522,549) and (b) the occasional RNAs that extend beyond the usual polyadenylation site (6,178,394). In contrast with the orderly transcription of intact cells are the random initiations experienced by more than one laboratory in nuclear run-off transcription assays late in infection (151,528). The abundance and stability of the various HSV-1 mRNAs appear to vary (136,137,467,469). In general, mRNAs of α and β genes appear more stable than those of γ genes (544). Viral mRNAs may persist in the cell after their translation ceases (210,241). Although complementary RNA sequences are readily detected in infected cells, double-strand RNA does not accumulate (197, 242).

The Environment of the Viral Genes

The open-reading frames identified to date are embedded, for the most part, in domains exhibiting both virus/host-common (e.g., TATAA boxes) and virus-specific 5′ sequences. The absence of TATAA boxes from some transcriptional units has been noted (see, e.g., ref. 57).

Studies on the structure of HSV genes have focused on two specific objectives. The first objective involved (a) the minimal promoter domain and (b) the *cis*-acting sites required for gene expression. The second objective was to identify the *cis*-acting sites that confer upon the target gene the capacity to be regulated as an authentic viral gene. Only a few HSV genes have been analyzed in sufficient detail to reveal and identify the *cis*-acting signals embedded in them. The most thoroughly studied, and the one that has generated the most conflicting results, is the *tk* gene. Also worthy of

discussion are the α4 gene (Fig. 9) and, to a much lesser extent, two representative γ_2 genes, although the level of controversy is not nearly as high.

In the α4 gene, the 5′ nontranscribed domain extending upstream from the cap site to nucleotide −110 is capable of imparting to a reporter gene the capacity to be transcribed efficiently in the absence of viral *trans*-activating factors (279,280,367). Other than the transcription initiation site, the *cis*-acting sites that affect expression have not been investigated in detail. The sequences upstream from nucleotide −110 confer on α and β promoters a higher basal level of expression as well as the capacity to be induced as α genes by viral *trans*-activating factors (245). The higher level of expression conferred by the sequences upstream from nucleotide −110 is very likely due to the SP1-binding sites embedded in G+C-rich inverted repeats which abound in that region (214,215,245,278–280). At least one sequence that confers inducibility as an α gene is the *cis*-acting site for αTIF [5′ NC GyATGn-TAATGArATTCyTTGnGGG 3′ (245,279)]. Separation of promoter and regulatory domains has been noted in other α genes. Although G+C-rich stretches are frequent, SP1-binding sites have not been reported in other α genes (Fig. 9).

ICP4-binding sites have been reported in the promoter-regulatory domain and across the transcription initiation site of the α4 gene, as well as in the promoter domain of the α0 gene, but not in the other α genes (120,246,247,328).

The initial analyses of the *tk* gene rested on the erroneous notion that it exemplified the structure of a typical eukaryotic gene. Only recently has there been an effort to understand its structure as a viral, β gene. Several features are of particular interest. The *tk* gene appears to have two transcription initiation sites, and mRNAs derived from both sites have been reported (98,375,549). The role of these sites in the expression of the gene in lytic infection is not clear. The 5′ nontranscribed region has been very thoroughly investigated by McKnight and co-workers (104,305–309) and by Silverstein and co-workers (108,109,142). The initial studies identified a 110-nucleotide region upstream from the site of initiation of transcription that was minimally required for efficient expression in the absence of viral *trans*-activating factors. This promoter contains a "proximal" *cis*-acting site and two "distal" sites, all of which are important for constitutive expression of the *tk* gene and probably function as punctuation sites and sites for binding of RNA polymerase and accessory factors (549). In subsequent studies a CCAAT box, two SP1-binding sites (214), and an octamer motif (ATTGCAT) upstream of −116 were also identified (350). Mutations in all of these sites affect expression of the uninduced gene. However, attempts to define a *cis*-acting site that is virus-specific have not been successful. The protocol employed involved studies of linker scanning mutations. Inasmuch as the authors failed to identify mutations which affected *trans*-activation but not expression in the absence of viral *trans*-activating factors, they concluded that the viral factors which *trans*-activate viral β gene

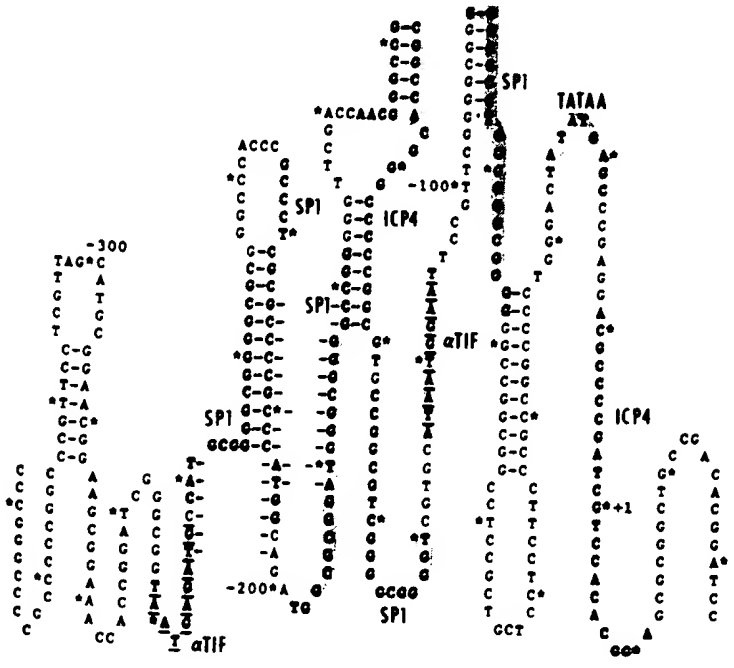

FIG. 9. Schematic representation of the structure of the 5′ nontranscribed domain of the α4 gene. An asterisk marks every 10th nucleotide. The transcription initiates at nucleotide +1. The shaded areas represent the binding sites of TATAA protein, SP1 and ICP4, and the host protein–αTIF complex. The sequence is shown in a folded form to emphasize the presence of the perfect G+C-rich inverted repeats that abound in this domain of the gene. In some instances, the G+C-rich regions can form alternate stem structures; these are identified by the dashes. (Data were taken from ref. 280.)

expression act on a host factor and not directly on DNA.

More recent studies have focused on the interaction of ICP4 with domains of the *tk* gene and on mutations downstream from the *tk* cap site. Notwithstanding earlier reports that ICP4 does not bind to the *tk* gene directly (97,403), the following facts are evident: The ICP4 does indeed bind to several domains of the gene both upstream and downstream from the cap site, and the binding sites include both (a) sequences similar to the consensus binding site reported by Faber and Wilcox (119) and (b) highly degenerate sites with little resemblance to the consensus (e.g., see ref. 316). Of particular interest are (a) the ICP4-binding sites in the transcribed noncoding domains and (b) the role of the transcribed noncoding sequences with respect to the regulation of *tk* as a β gene, as discussed below.

The structure of the γ genes, and particularly of the γ_2 genes, is the least well understood and is likely to be highly variable. Analyses of three genes—glycoprotein C, UL49, and US11—suggest that the sequences required for efficient expression include the TATAA box and extend into the 5′ transcribed noncoding domain (73,98,118,180,181,211–213,289,290, 458,466). The sequences required for the γ_2 regulation of these genes appear to also include sequences downstream from the TATAA box. Embedded in transcribed domains, as well as in the 5′ nontranscribed sequences, are ICP4-binding sites whose functions are not yet known (316).

Regulation of HSV Gene Expression: *trans*-Acting Factors

Given the complexity of herpesvirus multiplication, the reproducibility of the general pattern of virus multiplication long ago suggested that the events must be tightly regulated (413). Regulation of HSV gene expression has become a major focus of research, and the mechanism by which the virus regulates its replication remains an exciting area of investigation.

Turning on HSV genes involves several elements: (a) *cis*-acting sites for both viral *trans*-acting factors and cellular transcriptional proteins, (b) *trans*-acting signal proteins specified by the virus, and (c) both viral and cellular factors involved in viral DNA synthesis and in posttranslational modification of viral proteins. It is convenient to review the features of the cascade regulation of gene expression illustrated in Fig. 4 and described above by considering the *trans*-acting factors and their *cis*-acting sites.

α-*Trans*-Induction Factor (αTIF)

α genes are induced by a structural protein of the virus, αTIF. The *cis*-acting site (245,280)

5′ NC GyATGnTAATGArATTCyTTGnGGG 3′

binds a host protein (248,249) designated variously as OTF-1, NFIII, or αH1 (248,249,342). αTIF is packaged in 500–1,000 copies per viral particle (170). It does not interact with the viral DNA directly; rather, it binds to the complex formed by the host protein and the *cis*-acting site on viral DNA (145,303,336,374,507). The *trans*-activating function of the protein maps near its carboxyl terminus (438,506); its association with the DNA either as a chimeric DNA-binding protein or through αH1/OTF-1/NFIII, etc., is required for *trans*-activation. A characteristic of αTIF is that it is packaged in the virion tegument, and upon release of the intravirionic contents in the newly infected cell it is translocated to the nucleus independently of the viral DNA; that is, at nonpermissive temperature the mutant HSV-1(HFEM)*ts*B7 *trans*-activates α-regulated genes even though the viral DNA is not released from capsids (21).

The induction of α genes by αTIF presents numerous unresolved puzzles. Foremost, there is no explanation for the failure of the α genes to be expressed late in infection in light of the massive amounts of αTIF accumulating in the infected cells at that time. Exhaustion of functional host protein is a possibility, but the presence of OTF1/NFIII/αH1 capable of binding to DNA can be readily demonstrated in infected cells late in infection.

The second puzzle stems from the observation that the footprint of the host protein required for αTIF binding is in the sequence ATGnTAAT. The 5′ domain of this sequence is the least conserved domain of the *cis*-acting site, whereas the sequence TAATGArAT is highly conserved (280), suggesting either that αTIF makes contact with the DNA in the GArAT sequence or that another DNA-binding protein is also involved.

ICPO

The product of the α0 gene is predicted to be 80,000 in apparent molecular weight, but in denaturing polyacrylamide gels it migrates with an apparent molecular weight of 110,000–124,000, depending on the type of cross-linking agent used and on the acrylamide concentration. $\alpha 0^-$ mutants are viable in cell culture, and *ts* mutants have not been reported (437,489). In transient expression systems, ICPO has been reported to promiscuously *trans*-activate transfected genes by itself or in combination with ICP4 (114,117,142–144,337–339,358,391,440). Of the various experimental designs, the most convincing are those in which the *trans*-activation of target genes was done in conjunction with ICP4. The function of ICPO in infected cells is not known and is not readily apparent. The transient expression studies suggest that it may act as a *trans*-activator of the α4 gene. No *cis*-acting site is readily

apparent. Deletion mutants in the α0 gene grow in cell culture, albeit more sluggishly than the wild-type virus, particularly at low multiplicities of infection (437,489).

ICP4

This protein is predicted to be 132,835 in translated molecular weight (294). Most fresh HSV-1 isolates show *ts* α4 activity, and 37°C/39°C ratios of plating efficiencies as high as 10^6 have been recorded [e.g., HSV-1(F)]. It is then perhaps not surprising that *ts* mutants in the α4 gene have been readily isolated by a number of laboratories (371,372,521). ICP4 forms three bands (designated as 4a, 4b, and 4c) in denaturing polyacrylamide gels (357). In cells infected with wild-type virus, the fastest migrating band (band 4a) has an apparent molecular weight of 160,000 and is readily detected in the cytoplasm after pulse-labeling with radioactive precursors (183,184,357,539). It is also the only form accumulating in cells infected with certain α4 *ts* mutants and incubated at nonpermissive temperatures (233). Bands 4b and 4c have apparent molecular weights of 163,000 and 170,000, respectively, and accumulate in the nucleus (357,539). The accumulation of the slower migrating bands coincides with (a) translocation of the protein into the nucleus and (b) labeling with inorganic ^{32}P phosphate added to the medium (124,357). ICP4a and ICP4c can be pulse-labeled with ^{32}P during the reproductive cycle long after the synthesis of this protein ceases, suggesting that phosphate cycles during infection (539). Preston and Notarianni (376) reported that α4 accepts ADP ribosylation in isolated nuclei; more recent studies (J. Blaho, N. Michael, and B. Roizman, *manuscript in preparation*) indicate that ICP4 is poly(ADP)-ribosylated. ADP ribosylation could account for the labeling of ICP4 by inorganic phosphate from the medium (357), but phosphate also cycles on and off ICP22 and ICP27 (539); whereas, ICP4 appears to be the only α protein to be poly(ADP)-ribosylated.

ICP4 is the major *trans*-activator of HSV genes. Long a subject of study, it has generated a rich, passionate (and sometimes confusing) literature. $\alpha 4^-$ mutants grow only in cells expressing ICP4 proteins from a copy of the α4 gene embedded in the cellular genome. Fine anatomical dissection of the gene has outlined domains necessary for autoregulation of α genes, for the synthesis of proteins made later in infection, for phosphorylation, and for nuclear transport (95–97,189,351,471). It is convenient to consider the effects of ICP4 on α, β, and γ genes separately.

In the case of *ts* α4 mutants, both copies of the gene are mutated, as would be expected for the expression of the *ts* phenotype (233). The phenotypes of these mutants vary. At the nonpermissive temperature, some mutants express both α proteins and selected sets of proteins normally made later in infection (93). A most interesting group of *ts* mutants in the α4 gene overproduce α proteins at the nonpermissive temperature (93, 521). There is convincing evidence that ICP4 turns off its own synthesis and that this autoregulation correlates with the binding of the protein to a *cis*-acting site across the transcription initiation site of the gene (97,328,403). Comparisons of the α RNAs accumulating in cells infected and maintained at permissive and nonpermissive temperatures indicate that the α genes subjected to repression are primarily α4 and α0 (J. Hubenthal-Voss and B. Roizman, *unpublished studies*). These genes are the only ones in which a high-affinity ICP4-binding site corresponding to the consensus sequence ATCGTCnnnnCnGnn have been reported (246,247,328). As noted above, in the case of the α4 gene, the binding site ATCGTCcacaCgGag is across the cap site (328). In the case of the α0 gene, the binding site ATCGTCactgCcGcc is at position −64 to −49 (247). The correlation between binding activity of ICP4 to DNA and shut-off of α4 transcription, if confirmed in the case of α0 as well, would suggest that ICP4 can turn off transcription even when the binding site is upstream of the transcription initiation site.

The *trans*-activation of β genes has been discussed above. ICP4-dependent activation of transcription of a β gene embedded in the viral genome occurs from a very much lower level of basal expression than that seen from an isolated gene introduced into the same cells. After *trans*-activation, the level of *tk* gene expression is higher than that attained in cells transfected with the isolated *tk* gene. ICP4 DNA-binding sites in the domain of the *tk* gene both upstream of the cap site and downstream from nucleotide +50 have been demonstrated by several groups (246; Mavromara-Nazos and Roizman, *unpublished studies;* S. Silverstein, *personal communication*) but not by others (403). Studies by Halpern and Smiley (163) and by Mavromara-Nazos and Roizman (290) have failed to demonstrate a significant role of the binding to sites downstream of nucleotide +51.

It would seem to us that *trans*-activation of β genes involves two functions: (i) release from a repressive state and (ii) *trans*-activation. Since neither of these occurs at the nonpermissive temperature in cells infected with *ts* mutants in the α4 gene, then at least one, the initial event, depends on ICP4. To aficionados of transient expression it is worthwhile to point out that in cells transfected and selected for *tk* activity, the ratio of induced to basal TK activity after *trans*-activation with virus is considerably lower than that obtained in cells which are (a) transfected with a plasmid containing TK and another marker and (b) selected for the other, covalently linked marker (245). We interpret this to indicate the following: For a constant ratio of *tk*

genes per cell, the fraction of derepressed *tk* genes is higher in the cells selected for TK activity, but α4 derepresses *tk* genes in both systems. Inasmuch as binding of ICP4 to DNA correlates with repression irrespective of the position of the binding site, it may be useful to entertain the possibility that derepression may involve modification of a host protein rather than binding to DNA per se.

Earlier in the text we noted that as in the case of the β-*tk* gene, the γ genes are expressed and regulated differently in the context of the environment of the viral genome than in that of the cellular genome. The assessment of the role of ICP4 in the *trans*-activation of γ genes is complicated by several additional factors. Foremost, γ genes and especially γ_2 expression require viral DNA synthesis as a *cis*-acting function (289). Second, although it has been reported that the cap site and TATA box of the gene encoding US11 is all that is required for "fully efficient regulated activity" (211), the regulatory domains of at least two γ_2 genes appear to be downstream from the TATAA box and very likely include the 5′ transcribed noncoding domains (290). These domains contain ICP4-binding sites (316; P. Mavromara-Nazos and B. Roizman, *unpublished studies*). A possible role of α27 has also been noted, as described below.

Recently, two observations suggested a possible scenario for viral gene regulation: First, a chimeric gene consisting of 5′ nontranscribed domains of the β-*tk* gene and the 5′ transcribed noncoding domains of a γ_2 gene was expressed both early and late in infection (recombinant R3820), while a chimeric gene consisting of γ_2 5′ nontranscribed sequences and β-*tk* 5′ transcribed noncoding domains was barely expressed (recombinant R3821) (290). In addition, Arsenakis et al. (9) reported that the *gD* gene contained in a large HSV-1 DNA fragment was expressed in baby hamster kidney cells lacking the α4 gene but was not expressed in cells expressing significant amounts of ICP4 protein. One hypothesis (289) of at least heuristic value is that (i) the *trans*-activation of γ_2 genes requires removal, during DNA synthesis, of a transcription blocking factor and also requires *trans*-activation of transcription by another factor, (ii) the transcription blocking factor is effective only for transcriptional *trans*-activators operating downstream from the block and does not affect transcriptional activators acting upstream from the block, and (iii) the *cis*-acting sites for both the blocking factor and the transcriptional factor are in the 5′ transcribed noncoding domains. This hypothesis is consistent with the observations that (i) 5′ transcribed noncoding domains contain the *cis*-acting sites necessary for γ_2 gene expression, (ii) genes whose 5′ domains consist of the *tk* 5′ nontranscribed domains fused to the γ_2 5′ transcribed noncoding domains are regulated as both β and γ_2 genes, and (iii) in cells infected with $\alpha 27^-$ mutants, viral DNA is made but γ_2 genes are not expressed (i.,e. DNA synthesis alone is not enough to activate γ_2 gene expression). Does ICP4 block transcription, *trans*-activate it, or both? The evidence that, in cell-free systems, ICP4 increased the transcription of *gD* [a γ_1 gene (499)] is not in itself impressive, since (i) the ICP4-binding site tested was upstream from the reported minimal sequence required by gD to be regulated as a γ_1 gene [nucleotide −55; (113,115)], (ii) in transient expression systems, for what it is worth, late γ_2 gene expression was activated at low concentrations of the ICP4 gene but not by high concentrations of the gene, and (iii) the amount of ICP4 used in that study was arbitrary, without relevance to either known positive or negative *trans*-activation.

The hypothesis that ICP4 does not act directly on the DNA cannot be dismissed out of hand. Much has been made of the observation that α4 and the equivalent gene product of pseudorabies virus induce not only herpesvirus genes but also adenovirus and cellular genes (e.g., β-globin) introduced into cells by transfection (26,121,161). More recent studies have shown that the *trans*-activation of adenovirus late gene expression by the ICP4 equivalent of pseudorabies virus is through enhanced formation of transcription initiation complexes (1). While very revealing of a biased experimental design (the experimental design assumed *a priori* that the herpesvirus major regulatory protein *trans*-activates in a nonspecific fashion, since it is hardly likely that herpesvirus *trans*-activating proteins evolved to *trans*-activate adenovirus genes), these studies in fact do not contradict the evidence that ICP4 acts directly by binding to specific sites on the DNA. ICP4 appears to be a multifunctional protein. It could very well be that ICP4 and its homologues derive from a cellular transcriptional factor. Foremost, however, ICP4 has a dual function as a repressor and as a *trans*-activator; the function of the protein may well be determined by the position of its binding site, the strength of its binding to the DNA, and the nature of the posttranslational modification to which it has been subjected (316).

Other Factors Implicated in trans*-Activation of Viral Genes*

ICP8, ICP22, and ICP27 have been implicated in gene regulation.

The molecular properties of ICP8 were described earlier in the text. Most (but not all) of the effects of ICP8—namely, enablement of late gene expression and the effect of mutants on the expression of early genes—could be rationalized from its role in late DNA synthesis, but only because its role in DNA synthesis is known. To explain the shut-off of expression of viral

genes, it is necessary to postulate occlusion of transcription by overreactive ICP8 (151–153).

The predicted translated and apparent molecular weights of ICP22 are 46,521 and 72,000, respectively (183,184,294). Studies on a deletion mutant (368) indicate that the α22 gene is required in some cell lines but not in HEp-2 or Vero cells, presumably because the latter two cell lines express functions similar to that of α22 (450). In the nonpermissive cells (rodent cell lines and human cell strains), viral DNA is made, but late (γ_2) genes are not expressed efficiently.

The predicted apparent molecular weights of ICP27 are 55,249 and 58,000, respectively (183,184,294). Both *ts* and deletion mutants in the α27 gene have been reported (291,436). Notwithstanding a report to the contrary (281), the gene appears to be essential; cells infected with deletion mutants synthesize viral DNA, but late (γ_2) genes are not expressed (291; D. Knipe, *personal communication*). The phenotype of $\alpha 27^-$ viruses resembles that of mutants in the β8 gene (i.e., reduced synthesis of viral DNA, absence or reduced synthesis of γ proteins, and increased synthesis of β proteins). In transient expression systems, ICP27 has both positive and negative effects (116,376a,396,453,491a).

Posttranscriptional Regulation

The evidence for posttranscriptional controls is based on reports that translocation of viral transcripts into the cytoplasm appears to be regulated (216,218,241). Specifically, the genetic complexity of the RNA accumulating in the nuclei of cells infected with HSV in the presence of cycloheximide and maintained in medium containing the drug was greater than that observed in the cytoplasm. In retrospect, the interpretation of the data is not clear. The failure to demonstrate RNA complementary to β genes (e.g., *tk*) in nuclei of infected cells treated with cycloheximide (266) suggests that the transcripts accumulating in the nuclei might be random transcripts of the DNA rather than transcripts of specific genes belonging to the β and γ groups.

The evidence for translational regulation is based on several observations. Specifically, the inhibition of host protein synthesis by structural components of the virion soon after infection (125,333,395) and the inhibition of α gene product synthesis by subsequent gene expression (124,184) are translational events inasmuch as they occur in physically and chemically enucleated cells. A significant finding to emerge from the studies by Kwong and Frenkel (252) and Oroskar and Read (344) is that virion structural components exert an inhibitory effect on both host and α protein synthesis, inasmuch as mutants defective in the virion host shutoff function produce more α gene products than do their wild-type parents. It has been suggested that mRNAs of genes turned off in the transition from α to β to γ gene expression remain associated with polyribosomes. Studies by Johnson and Spear (210) reported the continued cytoplasmic accumulation of functional mRNA specifying glycoprotein D, a γ_1 polypeptide, after gD synthesis had declined.

HSV Gene Regulation: The Problems in Experimental Designs

In the preceding section we reported the conclusions that are supported by evidence. Examination of the literature of the past several years has disclosed three problems. Central to the evaluation of the available data are three highly significant, inescapable (and unfortunately inflammatory) issues.

The first is the evaluation of the methodology on which much of the data rests. The gold standard for the studies of viral gene regulation is the pattern of expression in productive infection of natural or reporter genes contained in the viral genome. Tests of modified *trans*- or *cis*-acting domains of individual genes are easier to perform and may, in some cases, be more meaningful if they can be done in the environment of the cell and in the presence of only a minimal amount of viral genetic information. However, the validity of such tests hinges on the extent to which they reproduce the regulation of the gene embedded in the viral genome and expressed in the course of viral infection. The expression of isolated α genes, in biochemically transformed cells or in transient expression systems, appears to mimic, to some extent, the regulation of the corresponding genes contained in viral genomes during productive infections (367). Notwithstanding the massive number of transfections which argue that ICP0 is a promiscuous *trans*-activator, supporting evidence from studies on deletion mutants in the α0 gene is not readily available. The transfection system apparently fails if more than two components of the regulatory pathway are introduced into the cell simultaneously—for example, the cotransfection of αTIF, α4, and the intended target gene of α4 (403). In the case of γ_2 genes, the transient expression system yields totally false results: Viral genes permanently integrated in cellular genomes or transiently expressed after transfection are regulated as β genes (10,28,466). The transfection system has given rise to a veritable cottage industry, but the results it has generated are not totally reliable. What is the evidence that viral genes other than those carrying the α-*cis*-acting sites can be regulated in that system in a mode which resembles viral gene regulation? If γ_2 genes are regulated as β genes, then what is the evidence that β genes in transfected cells are regulated as bonafide β genes?

The second issue centers on distinctions between experimental objectives and experimental design. It is intuitive that experimental designs should discriminate between alternative hypotheses and be able to predict a specific outcome that would unambiguously reject untenable alternatives. However, consider the following situation. As noted earlier in the text, the *tk* gene has been extensively used in transient expression systems for analyses of its *cis*-acting domains, and the gene is readily expressed in such systems. This is not the case for the *tk* gene contained in the viral genome and introduced into cells by infection. In this instance, in the absence of competent α proteins, the *tk* gene is not expressed. In the past several years a score of laboratories have introduced mutations into the viral genome in an attempt to define the *cis*-acting site for *trans*-activation of the *tk* gene by ICP4; not once has it been stated *a priori* what should be the phenotype of a *tk* gene whose ICP4 *cis*-acting site has been inactivated without also reducing the ability of the uninduced gene to be expressed! The unstated assumption is as follows: The ICP4 *cis*-acting site is distinct from that of the essential promoter sites, and in the absence of the *cis*-acting site for induction by ICP4 the *tk* gene would be expressed, albeit poorly. But how can this be true if in the absence of ICP4 the inherent capacity of the *tk* gene to be expressed is blocked? If viral proteins inhibit expression that is unblocked by ICP4, the only expected effect of mutations is full *tk* expression except when *cis*-acting sites required by the uninduced promoter are inactivated.

An additional point that seems to be ignored is that the *tk* gene is a late β gene; that is, its expression concurs with, rather than precedes, DNA synthesis as compared with ICP6 (the major component of ribonucleotide reductase) or ICP8 (the single-strand DNA-binding protein), which are abundantly expressed early in infection. In cells infected with wild-type virus, the expression of the *tk* gene is dependent solely on the parental genome, and progeny DNA does not appear to contribute significantly to the TK pool. Quite to the contrary, because the expression of β genes is reduced late in infection, inhibition of DNA synthesis enables larger amounts of TK to accumulate in the infected cells.

It would appear that the existing data do support the hypothesis that ICP4 *trans*-activates the *tk* gene by augmenting the effect of a host transcriptional factor, and therefore its *cis*-acting site is that of the host factor. However, the data also support the hypothesis that ICP4 merely releases the *tk* gene from a repressed state. In fact, experimental designs employed to date may also support additional hypotheses.

The last issue concerns the definition of the elements that play a role in the regulation of gene expression. In principle, we need to differentiate between two elements: (i) proteins whose function is to *trans*-activate one or more viral genes by acting on specific *cis*-acting sites and (ii) viral proteins which affect viral gene expression by a global action on the viral genome. αTIF and ICP4 are examples of bonafide regulatory proteins acting in *trans* on specific *cis*-acting sites. Clear examples of proteins which alter regulation by global effects on viral genomes are the single-strand DNA-binding protein and DNA polymerase. Malfunction of either protein (e.g., DNA$^-$ *ts* mutants at the nonpermissive temperature) would block the appearance of γ_2 proteins. In the case of the single-strand DNA-binding protein and the polymerase, the global effects can be readily substantiated by showing that they are part of the minimal set of genes required for amplification of viral DNA through its origin of DNA synthesis. We should note, however, that ICP8 has been implicated in many more regulatory events than can be ascribed to DNA synthesis. These effects could be due to nonspecific occlusion of transcription by overactive binding of ICP8 to single-strand DNA. The less clear situation concerns genes whose functions are not known and which affect viral gene expression. They may be bonafide regulatory proteins which affect transcription by acting on specific sites at or near the genes they *trans*-activate, or they may act globally in a manner which cannot be tested currently. The cases in point are the functions of the genes α27 and α22. Neither protein has been unambiguously shown to bind to viral DNA. Deletion mutants have shown that both genes affect late gene expression, selectively in some cells in the case of α22$^-$ and in all cells tested in the case of α27$^-$ viruses. Are they site-specific *trans*-activators, or are they proteins with global effects on the structure of the viral genome?

THE FATE OF THE INFECTED CELL

Cells productively infected with herpesviruses do not survive. Almost from the beginning of the reproductive cycle the infected cells undergo major structural and biochemical alterations that ultimately result in their destruction.

Structural Alterations

Changes in Host Chromatin

As described in detail elsewhere (410,417) and shown in Fig. 10, one of the earliest manifestations of productive infection is in the nucleolus; it becomes enlarged, becomes displaced toward the nuclear membrane, and ultimately disaggregates or fragments. Concurrently, host chromosomes become marginated, and later in infection the nucleus becomes distorted and

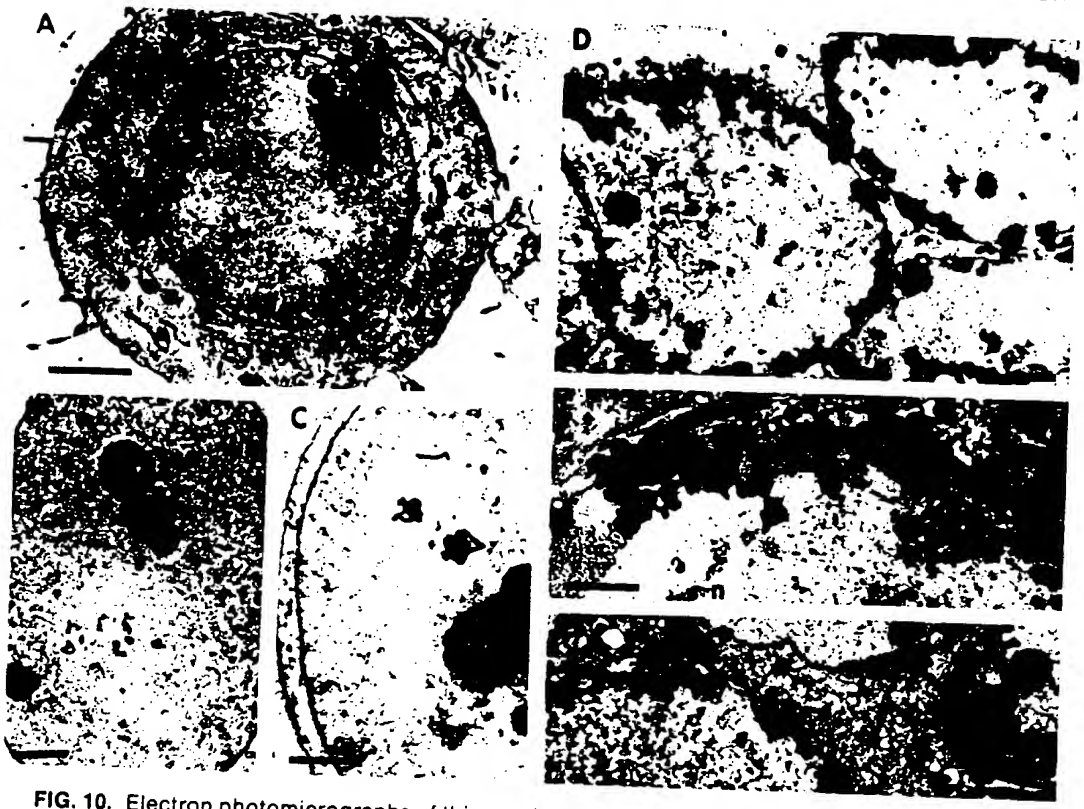

FIG. 10. Electron photomicrographs of thin-section autoradiography of HEp-2 cells infected with herpes simplex virus. **A:** A 4-hr-infected cell pulse-labeled for 15 min with [^{3}H]methyl thymidine prior to fixation. **B** and **C:** Enlargements of nuclei prepared as in part A. Note the disaggregated nucleolus. **D:** Portions of three nuclei of 18-hr-infected cells labeled with [^{3}H]methyl thymidine prior to infection. Unlabeled thymidine was present in the medium during and after infection. **E** and **F:** Electron micrographs of nuclei taken at higher magnifications. Note the aggregation of chromatin at the nuclear membrane. One of the cells in parts D and F did not synthesize DNA during the short labeling pulse. Abbreviations: n, nucleus; c, cytoplasm; v, aggregation of virus-specific, electron-opaque material. (From ref. 410 and from J. Schwartz and B. Roizman, *unpublished photomicrographs.*)

multilobed. The numerous protrusions and distortions have previously been mistaken for amitotic division (219,449). Margination of the chromosomes may or may not be linked with the chromosome breakage reported by numerous investigators (for review, see ref. 417).

Virus-Induced Alteration of Cellular Membranes

Changes in the appearance of cellular membranes (and, in particular, of nuclear membranes) are characteristic of cells late in infection. Deposition of material (tegument proteins?) on the inner surface facing the nucleoplasm or cytoplasm, but not in the space between inner and outer lamellae or cisternae of the endoplasmic reticulum, results in the formation of thickened patches along the membranes. Ultimately, the patches in the nuclear membrane coalesce and fold upon themselves to give the impression of reduplicated membranes (Fig. 5) (69,263,293,321,332,447,464).

The first inkling that herpesviruses modify cellular membranes was based on the observations that mutants differ from wild-type strains with respect to their effects on cells: Whereas wild-type viruses usually cause cells to round up and clump together, mutants may cause cells to fuse into polykaryocytes (105,407,408). These observations led to the prediction that herpesviruses alter the structure and antigenicity of cellular membranes—a prediction fulfilled by (a) the demonstration of altered structure and antigenic specificity (402,425) and (b) the presence of viral glycoproteins in the cytoplasmic and plasma membranes of infected cells (171,423,424,476).

The presence of gD in the plasma membrane of infected cells seems justifiable in light of the evidence cited earlier in the text, that gD precludes reinfection of cells with the progeny virus released from that cell.

gE and gI act as an Fc receptor (22,205,345,346). The function of the Fc receptor is not known. Several reports (60,138,452,470) indicate that gC binds the third component of complement, possibly in order to reduce the availability of the component for the immune lysis of the infected cell. Our knowledge regarding the processing, metabolism, and function of viral glycoproteins is not sufficient to deduce the reason for the presence of viral glycoproteins on the surface of the infected cell. The reason could be chance (a reflection of the natural flow of membrane-bound proteins) or design (the evolution of a pathway for cell-to-cell transmission of virus shielded from neutralizing antibody). Irrespective of the reason, the infected cell presents an altered antigenic specificity and becomes a target for destruction by the immune system.

The mutations (*syn*) which change the nature of the interaction of plasma membranes and affect the "social behavior of infected cells" have been mapped to several genetically unrelated locations on the HSV genome. Only one of these locations appears to map within the domain of a glycoprotein (gB) (282,434). Two other *syn* loci map within the UL24 and UL53 open-reading frames (89,196,364,366,434). A fourth locus has been mapped at the left terminus of the L component (UL1?) (269). All three predicted proteins contain hydrophobic regions suggestive of membrane proteins (294). This multiplicity of genes which affect cell fusion suggests that viral membrane proteins form interactive complexes and that alteration of any one component may alter the structure and function of the entire complex (434).

Host Macromolecular Metabolism

A characteristic of herpesvirus-infected cells is the rapid shut-off of host macromolecular metabolism early in infection. Thus, host DNA synthesis is shut off (Fig. 10) (421), host protein synthesis declines very rapidly, (252,395,414,494,495), host ribosomal RNA synthesis is reduced (518), and glycosylation of host proteins ceases (476).

HSV host shut-off occurs in two stages. The first stage, documented initially by Fenwick and Walker (125) and by Nishioka and Silverstein (333–335), involves structural proteins of the virus and does not require *de novo* protein synthesis. Thus, HSV shuts off host protein synthesis in physically or chemically enucleated cells (124); the shut-off was effected by density-gradient-purified virus but not by purified virus inactivated by heating or neutralization with antibody. The shut-off is faster and more effective in HSV-2-infected cells than in HSV-1-infected cells; this observation permitted the initial mapping of the genetic locus that confers upon HSV-1 × HSV-2 recombinants the accelerated shut-off characteristic of HSV-2 (122). More recently, isolation of *vhs* (virion host shut-off) mutants which fail to shut off host polypeptide synthesis in HSV-infected cells (395) has demonstrated more conclusively that this function is due to a virion protein (252,395).

The second stage, documented by Honess and Roizman (184,185), Fenwick and Roizman (124), Nishioka and Silverstein (334,335), and Silverstein and Engelhardt (468), requires *de novo* synthesis of proteins after infection. The shut-off coincides with the onset of synthesis of β proteins, but the experimental results do not exclude the possibility that the shut-off is caused by γ, rather than β, gene products.

Viral Genes Affecting Host Shut-Off

Structure and Expression of the vhs *Gene*

A rapid shut-off function was initially mapped to 0.52–0.59 (122). Isolation of a mutant defective in *vhs* function (395) allowed further mapping of the gene responsible. Mapping studies (253) have identified sequences from 0.604 to 0.606 on the viral genome as being responsible for the *vhs*$^-$ phenotype of the mutants. The open-reading frame in this region has been designated UL41, and it encodes a protein with an apparent molecular weight of 58,000 and a predicted molecular weight of 54,914 (294). A single mRNA has been shown to cross the minimal region shown to contain the mutations, 1.6 Kb in length with no introns (139). The RNA is expressed as a γ_1 gene.

Function of the vhs *Gene Product*

Early studies showed that virion components were responsible for destabilization and degradation of host mRNA (125). Further studies have shown that the virion component required for both mRNA destabilization and degradation is the *vhs* gene product. Furthermore, the *vhs* gene product is also responsible for a nondiscriminatory destabilization and degradation of viral α, β, and γ mRNAs (123,252,253,344,443,491). In cells infected with the *vhs*$^-$ mutant, host protein synthesis is not shut off. α and β protein synthesis are somewhat prolonged compared to wild type. Both of these effects have been shown to be due to a stabilization of host and viral mRNAs; in cells infected by *vhs*$^-$ mutants, mRNAs are not degraded as rapidly as in cells infected by wild-type virus.

Frenkel and associates (252) suspected that this function confers at least two advantages on the virus. First, it removes preexisting host mRNA from the pool of translatable messages, allowing the viral mRNAs to take over the pool rapidly. Second, destabilization of viral mRNAs allows a rapid transition from one regulatory class to the next. In the absence of the *vhs*

function, α and β proteins are produced beyond the time spans normally seen; the positive transcriptional controls discussed in the rest of this chapter are not enough to ensure efficient α-to-β and β-to-γ transitions. Although the *vhs*$^-$ mutation is not lethal, wild-type virus does have a growth advantage in tissue culture, indicating that efficient separation of the regulatory classes is helpful to the virus (252,395).

ICP47

The predicted translated and apparent molecular weights of ICP47 are 9,792 and 12,000, respectively. The gene appears to be nonessential for growth in cell culture. The α47$^-$ mutant phenotype apparent to date is the conservation of a host protein capable of binding with a high degree of specificity to a viral RNA transcribed across ori_s. In HSV-1-infected cells, the disappearance of this RNA-binding function is specifically associated with the production of functional ICP47 early in infection (428).

VIRULENCE

In healthy nonimmunocompromised humans, encephalitis occurs rarely (531). In experimental animals, it is frequently the only manifestation of disease. In studies on the molecular basis of disease induced by HSV, the endpoint of the research objective—the disease—is often synonymous with the destruction of the central nervous system (CNS). However, *neurovirulence*, as measured by intracerebral inoculation of virus, is clearly a misnomer. Wild-type HSV strains invariably multiply when injected into the CNS of experimental animals. Direct injection of virus into the CNS measures the capacity of the virus to grow and destroy an amount of CNS tissue that will result in death before the immune system blocks further virus spread. Because, in most instances, destruction of the CNS and death are related to virus multiplication (in quantitative terms), the CNS tissue specific growth, or *neurogrowth*, is measured in terms of the amount of virus required to reach a specific endpoint of tissue destruction.

A more rigorous attribute of virulence is invasiveness—the capacity to reach a target organ from the portal of entry. To disseminate to the target organ, it may be necessary for the virus to multiply at peripheral sites. In experimental systems, neurovirulence, the model of the disease producing the phenotype of HSV, is the consequence of (i) peripheral multiplication, (ii) invasion of the CNS, and (iii) growth in the CNS. Peripheral growth and invasiveness into the CNS can be quantified by measuring the amount of virus recovered (a) at the peripheral site and (b) in the CNS as a function of inoculum delivered to a peripheral site (i.e., footpad, eye, ear, etc.). The components of neurovirulence are not readily differentiable. *Neuroinvasiveness* can be differentiated from neurogrowth only in cases where the virus is capable of multiplying in the CNS (e.g., low PFU/LD_{50} ratios after intracerebral inoculation of mice) and at peripheral sites but incapable of invading the CNS. Failure of the virus to grow in the CNS abolishes its virulence, but the loss of tissue-specific growth is quite distinct from the capacity to invade the CNS.

Wild-type isolates differ with respect to neurogrowth and neuroinvasiveness: In our experience, isolates from the brains of encephalitis patients require the lowest pfu/LD_{50} ratios, as assayed by intracerebral inoculation in mice, whereas careful studies by Whitley and collaborators failed to differentiate between peripheral and CNS isolates from the same patients (R. J. Whitley, *personal communication*). Strains with elevated pfu/LD_{50} ratios yield mutants with increased neurogrowth, and these can be readily selected by serial passage in the mouse brain. Mutants with increased neuroinvasiveness can also be selected by serial passage of virus isolated from the brain but inoculated at a peripheral site. It is our impression that this heightened neuroinvasiveness is, to some extent, inoculation-route-specific.

Cell culture correlates of neurovirulence do not exist. No differences are readily apparent in cell culture among wild-type viruses differing with respect to neurogrowth by a factor of 100 (i.e., between approximately 1 and 100 pfu/LD_{50}). Some mutants requiring 10^6 pfu/LD_{50} may have a more restricted host range, reduced yield, or sluggish or multiplicity-dependent growth in cell culture (312,526).

In the past decade, virulence loci comprising both neurogrowth and neuroinvasiveness, or only one of these, have been ascribed to several sites, but particularly in or around the domain of the *tk* gene (126,312,381,481) and at the right terminus of the unique sequences of the L component in the P arrangement of HSV DNA (52,199,201,235,429, 502,503). At the *tk* locus, the neurovirulence may also be associated with genes other than *tk*, inasmuch as restoration of the *tk* gene in a *UL24*$^-$ mutant at another site did not result in increased capacity to cause death (B. Meignier and B. Roizman, *unpublished studies*). The major problem faced by most of these reports is as follows: Nearly any mutation or deletion introduced spontaneously or by design into the HSV genome results in reduced neurogrowth, and nearly any mutation or deletion introduced by default results in decreased virulence. Deletions and base substitutions in some genes have a more profound effect than those in other genes. For example, deletion of nearly any gene in the S component resulted in reduced capacity to grow in the CNS (312,526), and mutations in the ribonucleotide reductase subunit genes resulted in the same reduced capacity (42). It is obvious that any mutations (includ-

ing those that occur spontaneously and accumulate in laboratory strains characterized by a long history of serial passages in cell culture) and deletions that reduce the capacity to grow peripherally or in the CNS, or that reduce the capacity to invade the CNS, could be designated as "virulence loci," since rescue of the mutation or deletion will restore the phenotype of the parent virus [see, for example, the deletion in HSV-1 strain HFEM (235,429)]. Obviously, the genes that have been deleted or mutated are required for tissue-specific growth; their function is required, but is not sufficient, for neurovirulence in the context of the definition given here.

Also in the context of the definition of neurovirulence, none of the HSV genes tested to date, with the possible exceptions of gC and gE (52,207,312), appear to be dispensible with respect to replication in the CNS; this may also be true for other tissues. Genes specific for neuroinvasion have been reported, but these studies do not fully meet the stringent criteria for differentiation of neuroinvasiveness from failure to multiply efficiently.

It should be stressed that virulence is a multifactorial phenomenon reflecting the capacity of the virus to produce high yields and to spread in direct competition with the immune response whose objective is to block virus multiplication. Mutations to increased neurogrowth very likely enable (a) higher expression of viral genes whose functions are not complemented in the CNS and (b) more efficient molecular interactions of the viral genome or its gene products with the cellular factors required for viral replication. A central question is, Why do mutations for accelerated growth or for greater neuroinvasiveness not arise, since such mutants can be readily produced in the laboratory by serial passage?

Little is known of the mechanism by which HSV invades the CNS of human adults, whether by infection of a cranial nerve or by postsynaptic transmission. In principle, the epidemiologically significant virus in human-to-human transmission is that which appears on the mucous membranes or skin after first infection or after reactivation of latent virus. Although we have alluded to the observation that isolates from CNS tend to be virulent, selection for increased neuroinvasiveness is not a useful property inasmuch as the virus that multiplies in the CNS is less likely to be transmitted from person to person than by virus multiplying in the mucous membranes of otherwise healthy people.

LATENCY

The ability of HSV to remain latent in the human host for its lifetime is the unique and intellectually most challenging aspect of its biology. The virus enters sensory nerves innervating the cells infected at the portal of entry. In latently infected neurons, the viral genome acquires the characteristics of endless or circular DNA (133,315,404,405), may be in nucleosomal form (100), and has been reported not to be extensively methylated (103). To our knowledge, no functions are expressed which are required for the establishment of the latent state. In a fraction of those harboring HSV in a latent state, the virus is periodically reactivated; infectious virus is carried by axonal transport (70), usually to cells innervated by the infected neurons at or near the portal of entry (49,79,156,409). Depending on the host immune response, the resulting lesion may vary considerably in severity, from barely visible vesicles to rather severe, debilitating lesions in immunosuppressed individuals. The clinical aspects of latent infection and reactivation are discussed in Chapter 66. This section concerns the molecular biology of latency.

HSV Latency in Experimental Systems

The Experimental System

The most useful model systems are mice, guinea pigs, and rabbits. In the mouse, latent infection is readily established after eye, footpad, or ear inoculation, but the latent virus does not reactivate spontaneously (29,30,173,174,484). Latent virus in the rabbit does reactivate spontaneously (331). The guinea pig shows recurrent lesions after vaginal infection with high doses of HSV-2, but it is not clear whether these are the consequence of a festering, chronic infection or of a reactivated, truly latent virus (480). At the other extreme are latency models in cells cultured *in vitro*. The latently infected neuron is nonpermissive at the time it harbors the virus in the latent state. In the ganglion, the permissivity of the infected neuron is transient. When placed in culture, neurons become permissive. Those that contain latent virus activate its multiplication. It has been reported that neurons retain virus in a latent state in the presence of neuronal growth factor, and, conversely, the virus is activated when the growth factor is withdrawn (538). The issue is whether virus activation coincides with incipient neuronal death and whether permissivity for virus growth and maintenance of physiologic integrity are mutually exclusive.

A number of laboratories have reported the maintenance of the viral genome by rendering cells nonpermissive by a variety of methods (65,66,430,533–537). To our jaundiced eye, the state of nonpermissivity induced in cells in culture by elevated temperatures, interferon, or antiviral drugs is not equivalent to the nonpermissive state of the neuronal cells *in vivo*.

The events transpiring in animal models can be di-

vided into several stages. In the initial stage, virus replication ensues in the tissues at or near the site of inoculation. This initial multiplication ensures contact with, and entry into, the sensory nerve endings. The capsid is transported by rapid retrograde axonal flow to the neuronal nucleus (244,276). Data obtained from infection of neurons cultured *in vitro* indicate that the viral capsids are transported to neuronal nuclei by retrograde axonal transport involving microtubules. Drugs which disrupt neuronal microtubule structures, or which are known to inhibit retrograde transport of certain compounds, also inhibit the ability of the virus to move from the peripheral endings of neurons to the nuclei (244). Electron-microscopic studies indicate that in neurons infected in cell culture, the viral particle that is being transported is the unenveloped capsid (276).

We suspect that the initial multiplication is (a) not essential if the virus comes in contact with nerve endings and (b) critical if the virus is merely deposited on the surface of the peripheral tissues.

In some animal models, there is a short period of viral replication in the ganglia at this stage (226–230,301,302,382,383,508,509,520,542); however, this may be an artifact of the large amount of virus used in the inoculum to attain a high percentage of latently infected ganglia.

In the second stage, at a maximum of 2–4 weeks after inoculation, no replicating virus can be detected in the sensory ganglia innervating the site of inoculation.

In the last stage, certain stimuli (e.g., physical or emotional stress, peripheral tissue damage or intake of certain hormones in humans, and both peripheral tissue damage and administration of drugs that stimulate prostaglandin synthesis in experimental animals) may result in activation of virus multiplication concurrent with axonal transport of the virus progeny, usually to a site at or near the portal of entry. Although the issue is still being debated frequently and hotly, there is little doubt that virus multiplication results in destruction of the neuronal cell.

Viral Gene Expression in Latently Infected Neurons

Extensive studies on ganglia harboring latent HSV have been rewarded by an extreme paucity of evidence for viral gene expression. The only transcript detected to date is one designated optimistically as latency-associated transcript 1 (LAT1) (486). This transcript is abundant and accumulates in the nuclei of neurons of latently infected animals and humans (243,406,483, 485,486). LAT1 is spliced (517,527), and in latently infected cells it is not polyadenylated (516). It has been reported that the LAT1 population is heterogeneous, varying in the donor/acceptor splicing sites, and hence capable of expressing more than one protein—provided, of course, that it were transported to the cytoplasm (517,527). Because it is, in part, complementary to the 3′ terminus of the α0 mRNA, it has been thought that the function of LAT1 is to preclude the expression of α0 (486). Confounding the issue is the observation that *LAT1*$^-$ mutants are capable of establishing latency (200).

In a different category is the observation that in trigeminal ganglia harboring latent virus, there are between 0.1 and 1 viral genome equivalents per cell genome (40,384,404,405). This datum poses an intriguing question. Heretofore, the number of neurons harboring virus was thought to be between 0.1% and 3% of total neurons. Even assuming a 10% total, the number of neurons harboring virus would constitute less that 1% of all ganglionic cells. To account for the high number of viral genomes per cell harboring latent virus, it is necessary to postulate the following: (i) More than one viral genome can enter and establish latency in the same neuron, or (ii) viral genomes are amplified by the cellular machinery during the latent state (422).

The Role of Viral Multiplication in the Establishment and Assessment of the Latent State

There are several important facets of latent infections which relate to the role of virus multiplication, both at the periphery and in the neurons harboring the virus.

1. As noted above, HSV must have access to the nerve endings in order to establish latency, and therefore it could be expected that the greater the number of peripheral cells that become infected and support virus multiplication, the larger the number of neurons which will harbor latent virus. The relevant phenomenon in humans is that the frequency of reactivations resulting in recrudescences of lesions is related to the severity of lesions caused by the first infection. In the model we have proposed (422) and have elaborated below, the frequency of recurrences would be determined, in part, by the number of neurons harboring virus.

2. Several years ago, it was proposed that the latent virus makes a "round trip"; that is, the reactivated virus reestablishes the latent state by infecting the nerve endings of hitherto uninfected neurons (225). This hypothesis is not tenable. First, in experimental systems, it is very difficult to superinfect ganglia harboring latent virus with a second, marked virus (51,314). Perhaps even more significant, the "round trip" does not appear to take place in humans even under conditions that would favor such a phenomenon. Thus, in a small number of individuals, mutants that

were both virulent and acyclovir-resistant have arisen (110,349). Recurrent lesions that emerged after the mutant was eliminated with the aid of other drugs did not contain the acyclovir-resistant virus. While this phenomenon has been attributed to rapid elimination of the peripheral infected cells by the immune system (51), the observations may have more profound implications inasmuch as induction of latent virus should eliminate it from the ganglion.

3. The operational definition of latent virus is useful but self-limiting, in a rather significant fashion. Currently, latent virus is defined as that which is detected after incubation of intact ganglionic tissue with suitable susceptible cells and not by inoculation of the susceptible cells with macerated ganglia. While adherence to this operational definition is critical, in experimental animals and in humans, viral DNA can be detected in CNS tissue (particularly in brain stem), but infectious virus cannot be reactivated from these tissues. It has been customary to ignore these genomes or assume that the brain stem accumulates defective genomes. The possibility that this is not the case, that the neuronal population of the brain stem represents a population that is nonpermissive, must be considered in light of two observations. Foremost, reactivations are more readily demonstrable with so-called "virulent" (or, by the definition above, capable of heightened neurogrowth) strains than with relatively avirulent strains. Of greater potential significance is the recent observation that certain deletion mutants are not reactivated. While failure of $\alpha 0^-$ mutants to reactivate from neurons of ganglia known to contain viral DNA has been attributed to a key role of the $\alpha 0$ gene in reactivation (264), similar results have been seen with tk^- viruses (see below). Competence to reactivate may reflect a myriad of functions that include both those that are specific to the termination of the latent state and those related to the overall capacity of the virus to multiply in an extended host range—the relatively nonpermissive sensory neuron. These two sets of functions may be difficult to differentiate.

Establishment and Maintenance of the Latent State: The Data

Viral Gene Expression Required for the Establishment of Latency

The viral genome during latency has been reported to be in an "endless" form, either concatemeric or circular (i.e., in a state similar to that seen immediately after infection) (404,405). In cells in culture, acquisition of the circular or concatemeric form immediately after infection does not require *de novo* protein synthesis (362), and therefore the presence of circular DNA does not imply viral gene expression.

Reactivation of virus, as noted above, is not a reliable indicator of the ability to establish latency. If we accept the operational definition that the presence of LAT1 RNA is indicative of the latent state, it follows that any virus capable of infecting neurons is able to establish latency. To date, all HSV mutants (except those with deletions in the LAT1 sequence) shown to be capable of peripheral replication also appear to induce LAT1 in neurons of ganglia which innervate the site of inoculation (e.g., 63,312,313). Since mutants in all HSV genes have not been tested, the conclusion that no viral gene function is specifically required for the establishment of latency may be prognostic but premature.

Viral Gene Expression Required for the Maintenance of Latency and for the Activation of Viral Multiplication

Studies on the viral gene expression required for maintenance of the latent state or for activation of viral multiplication suffer from a peculiar operational problem: Inactivation of a gene essential for either process should result in failure to reactivate. Failure to reactivate virus from ganglia of experimental animals which had been inoculated with adequate amounts of virus by an appropriate route could be due to (i) failure to establish latency, (ii) failure to maintain the latent state, or (iii) failure of the latent genomes to be induced. Detection of viral genomes or of LAT1 in neurons eliminates, but does not discriminate between, alternatives (i) and (ii), but it introduces another alternative—namely, that the viral DNA retained in the ganglia and expressing LAT1 represents defective genomes. While appropriate experimental designs can surmount this problem, this has not been done so far.

Viruses which are capable of independent replication and which failed to be reactivated readily in mice are tk^- and $\alpha 0^-$ mutants. The ability of tk^- virus to establish latency has been disputed. While Tenser and colleagues (500,501) have reported that tk^- viruses cannot establish latency in mice and have ascribed a significance to that finding, other workers have reactivated viruses with little or no TK activity from mice, and even deletion mutants have been reactivated from latently infected rabbits (50,157–159,312,451). $\alpha 0^-$ mutants may fall into a similar category. Although they are not readily inducible in mice (264), viral DNA was detected in ganglia (264), and spontaneously reactivated virus was isolated from a rabbit (Y. J. Gordon, *personal communication*). The LAT1 RNA can be detected in neurons of mice infected with $\alpha 0^-$ (D. M. Knipe, *personal communication*) or tk^- (63) viruses.

As noted earlier in the text, the ability to reactivate may well reflect the ability of the virus to grow in re-

strictive cells. In experimental animal systems, there is the added requirement that viruses multiply effectively and rapidly, since viral multiplication and dissemination of the reactivated virus is in competition with the immune systems whose object is to ablate it. Examination of the results of reactivation from trigeminal ganglia of deletion mutants inoculated into mice is consistent with the hypothesis that mutations that affect growth of virus in cells also affect the ability of the mutants to be reactivated to a level high enough to be detected.

Reactivation of Virus in Experimental Systems

In humans, latent virus is reactivated after (a) local stimuli such as injury to tissues innervated by neurons harboring latent virus or (b) systemic stimuli such as physical or emotional stress, menstruation, hormonal intake, and so on, which may reactivate virus simultaneously in neurons of diverse ganglia (e.g., trigeminal and sacral). In experimental systems, induction of latent virus multiplication has been induced by (a) physical trauma to tissues innervated by the neurons harboring virus (8,165), and (b) iontophoresis of epinephrine (250,251) or other drugs (157,166,463). The molecular basis of reactivation, as well as the order in which viral genes are induced, is not known.

Establishment and Maintenance of the Latent State: A Model

The molecular basis of latency rests on answers to several key questions: (i) Since HSV readily multiplies in a variety of cells derived from human or animal tissues, why does lytic infection not ensue in neurons harboring latent virus? (ii) At what stage in the reproductive cycle is viral multiplication arrested? (iii) What is the origin of the rather high number of copies of the viral genome per latently infected neuronal cell? (iv) Why are all neurons not reactivated at the same time? (v) At what stage in the cascade of viral gene expression does replication of latent virus begin? (vi) Why is HSV-2 more readily reactivated in sacral ganglia, whereas HSV-1 is more readily reactivated from trigeminal or cervical ganglia? Our model is based primarily on the hypothesis that latency is required for the perpetuation of the virus in its natural host population and that the virus has evolved elaborate mechanisms to control the latent state.

The model we propose, largely for its heuristic value, is an extension of the one we described earlier (422) and consists of several components:

1. The model proposes that two distinct mechanisms operate to block the expression of HSV in neuronal cells. Thus, previously published data indicate that αTIF and capsids reach the nucleus independently inasmuch as viral mutants which do not release DNA at the nuclear pore do induce a reporter gene linked to an α promoter (21). The separation of the viral DNA from the *trans*-acting factor that induces the α genes is difficult to explain except in the context that it might be desirable for the virus not to multiply under certain conditions. Unlike the epithelial cell at the portal of entry with a distance of 10 μm or less between the plasma and nuclear membranes, in the infected sensory cells the distance between nerve endings and the nuclear membrane may well be measured in centimeters. In the absence of the αTIF, gene expression may not occur, or it would be grossly retarded. The absence of αTIF may not be sufficient to enable the the infected neurons to block viral expression. A second mechanism would be predicted to block the expression of α genes.

2. The model also proposes that activation of virus multiplication is the consequence of the cumulative effect of stimuli to which each cell harboring virus responds independently. Specifically, the hypothesis envisions that both local and systemic stimuli cause the viral DNA copy number to be increased. Virus would become activated when the copy number exceeds a certain threshold. Since the effect of the stimulus, the increase in the DNA copy number, and the precise threshold may vary from cell to cell, not all cells would be activated simultaneously.

3. The increase in the DNA copy number may not, per se, ablate the block in virus multiplication. The added requirement for viral multiplication falls under the heading of capacity for gene expression and is poorly defined. A common feature of this property is seen in the case of deletion mutants (e.g., $\alpha 22^-$; see ref. 450) infecting nonpermissive or restrictive cells. Such mutants often exhibit a multiplicity dependence reflected in the failure of virus multiplication at low, but not high, multiplicities of infection. The additional functions that may be required to achieve clinically detectable reactivation (or detectable amounts of infectious virus in experimental systems) may be those of a set of genes. The model proposes the following: (a) This set would include genes that are required, as well as those that are dispensable, for multiplication in cells in culture, and (b) the expression rate and product abundance of these genes would determine whether infection is productive or abortive in the particular cell in which the virus is latent.

HSV-1 and HSV-2 are very closely related viruses which have predilections for oral and genital mucosa, respectively. It could be argued that the differences in the nucleotide sequences of their genomes and in the amino acid sequences of their proteins reflect the differences in the environments in which they function. Conversely, the differences in the reactivation rates of HSV-1 and HSV-2 in trigeminal and sacral ganglia

must reflect the differences in the respective ganglia. The differences may be reflected not in the ability to establish latency but in the gene expression required to overcome the block to effective viral gene expression.

CONCLUSIONS

The studies on herpes simplex viruses are, at last, entering a most exciting stage, largely because the words "structure and function" are beginning to have an operational meaning. As a field of endeavor, we are beginning to characterize the interaction of proteins among themselves and with viral nucleic acids. In addition, the host factors crucial to virus multiplication, and potentially to latency, are being sought out. The armamentarium for a major assault on the mysteries underlying the biology of these viruses is in place, reflecting the contributions of many laboratories over many years.

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